Studies on transmissible spongiform encephalopathies - heterologous expression of PrP

Athina Voulgari

PhD

University of Edinburgh

1996

Declaration

I declare that this work is my own, unless otherwise stated in the text.

Signed

Athina Voulgari

Date 16/4/96

Acknowledgements

I would like to thank my supervisors, Peter Estibeiro, Rick Lathe and Jim Hope for their advice and for giving me the opportunity to come to Edinburgh.

I would like to thank all the staff of the Centre for Genome Research for their help and support.

Thank you to the Darwin Trust, the BBSRC and the Centre for Genome Research for sponsoring me.

I would like to thank John Bishop for his support and (extremely) helpful advice at the final stages, and proof-reading of the thesis.

Special thanks to Tom Gardner for his encouragement, sympathy, 'tuition', discussions, support, helping out at anytime with anything, massive amounts of photography +.....spicy stir fries.....

I would like to thank all my friends in Edinburgh (especially Glen, Tom D., Stamatis and Mandy) for their moral support, the "fun times", for trying their best to keep me entertained, chearing me up during the 'hard times' and.....taking my mind off the troubled thesis! Thanks to the Choreography Collective and my extra-curriculum activities I remained sane..... and had a great time.

Also thank you to all my Greek friends (especially Κατερινα, Μαρια, Μαρινα and Κωστας) for their invaluable support and encouragment and for visiting me in this foreign land!

Above all, I would like to thank my parents for....being my parents!

Για την Καιτη, το Θεμη και το Μιχαηλ

Glossary of Terms and Abbreviations

Units, measurements, physical and chemical quantities

c	10^{-2}	centi
m	10^{-3}	milli
µ	10^{-6}	micro
n	10^{-9}	nano
p	10^{-12}	pico
f	10^{-15}	femto
a	10^{-18}	atto
k	10^{3}	kilo
M	10^{6}	mega

m	metre
cm	centimetre
g	gram
µg	microgram
Da	dalton
kD	kilodalton
l	litre
ml	millilitre
µl	microlitre
v/v	volume/volume
w/v	weight/volume
s	second
min	minute
h	hour
A	ampere (current)
V	volt (potential)
Ω	ohm (resistance)
J	joule
°C	degree Celsius
M	molar
cpm	counts per minute
Ci	curie (1 Ci=37 GBq, 1 Bq=60 dpm)

rpm	revolutions per minute
u	units
OD	optical density

DNA (and related)

A	adenosine
C	cytidine
G	guanosine
T	thymidine
U	uridine
I	inosine
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
cDNA	complementary DNA
rRNA	ribosomal RNA
RNase	ribonuclease
DNase	deoxyribonuclease
dNTP	deoxy nucleotide triphosphate
NTP	nucleotide triphosphate
(d) (A/T/C/G) (T/D)P	(Deoxy) (base) (tri/di)phosphate
cAMP	cyclic adenosine monophosphate
nt	nucleotide(s)
oligo	oligodeoxyribonucleotide
poly(A)	polyadenylat(-e, -tion) polyadenylic acid
bp	base pair(s)
kb	kilobase(s)
aa	amino acid(s)

Klenow	DNA polymerase I, large fragment
Taq	Taq polymerase (heat-stable DNA polymerase)

Ala	alanine
Arg	arginine
Asn	asparagine

Asp	aspartic acid
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Iso	isoleucine
Leu	leucine
Lys	lysine
Met	methionine
Phe	phenylalanine
Pro	proline
Ser	serine
Trp	tryptophan
Tyr	tyrosine
Val	valine

Other chemicals, materials

ab	antibody
amp	ampicillin
APS	ammonium persulfate
BES	N,N-bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid
BSA	bovine serum albumin (fraction V)
CAT	chloramphenicol acetyltranferase
DAB	3,3' diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DLPC	phosphatidylcholine (L-α lecithin) solution
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
IPTG	isopropyl ß-D-thiogalactopyranoside

MOPS	3-[N-morpholino]propanesulphonic acid
MU	4-methylumbelliferone
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCA	phenol-chloroform-isoamylalcohol
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIPLC	phospholipase C
PK	proteinase K
PMSF	phenylmethanesulfonyl fluoride
RT	reverse transcript(ion/ase)
RT-PCR	reverse transcription coupled to polymerase chain reaction
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TBE	Tris-borate electrophoresis buffer
TE	10 mM Tris.HCl (pH 7.4)/1 mM EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TESPA	3-aminopropyltrioxysilane
Tris	tris(hydroxymethyl)methylamine
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Other

Aß (ß-AP, or ß/A4)	ß-amyloid peptide
ACE	adenylation control elements
AD	Alzheimer's disease
APP	amyloid precursor protein
BAC	bacterial artificial chromosome
BSE	bovine spongiform encephalopathy
C. elegans	Caenorhabditis elegans
CJD	Creutzfeldt-Jakob disease
CMV	cytomegalovirus
hCMV	human cytomegalovirus

CNS	central nervous sytem
CoA	coenzyme A
CPEs	cytoplasmic polyadenylation elements
CTA	clinical target areas
E. coli	Esherichia coli
$GABA_A$	γ-aminobutyric acid type A
GAG	glycosaminoglycans
GFAP	glial fibrillary acidic protein
GPI	glycosyl-phosphatidylinositol phospholipid
GSS	Gerstmann-Sträussler-Scheinker syndrome
CWD	chronic wasting disease
DY	drowsy
FFI	fatal familial insomnia
FSE	feline spongiform encephalopathy
HNE	HindIII-NotI-EcoRI
HY	hyper
hygro	hygromycin
Ig	immunoglobulin
ip	immunoprecipitation
IRE	iron responsive element
IRE-BP(IRF)	iron responsive binding protein
lacZ	ß-galactosidase
LCR	locus control region
L-DOPA	L-dihydroxyphenylalanine
LRS	lymphoreticular system
LTP	long-term potentiation
MAR	matrix-attachment region
MCS	multiple cloning site
NNE	non-neuronal enolase
neo	neomycin
NSE	neuron specific enolase
ORF	open reading frame
PAB	poly(A)-binding protein
PNS	peripheral nervous system
PrP	prion protein
(HaPrP	hamster PrP
HuPrP	human PrP

MoPrP	mouse PrP)
PrP^{c}	cellular prion protein
PrP^{sc}	proteinase K resistant prion protein
Prn-i	prion-incubation time gene
PRNP	human PrP gene complex
Prn-p	mouse PrP gene complex
Prn-p^{a}	mouse PrP gene allele a
Prn-p^{b}	mouse PrP gene allele b
RML	Rocky Mountain Laboratory
RRL	rabbit reticulosyte lysate
SAF	scrapie-associated fibrils
Sinc	scrapie incubation gene (in mice)
$Sinc^{s7}$	scrapie incubation gene allele s7 (in mice)
$Sinc^{p7}$	scrapie incubation gene allele p7 (in mice)
Sip	scrapie incubation gene (in Cheviot sheep)
Sip^{sA}	scrapie incubation gene, allele sA (in Cheviot sheep)
Sip^{pA}	scrapie incubation gene, allele pA (in Cheviot sheep)
SV40	simian virus 40
Tg	transgenic
TH	tyrosine hydroxylase
TK	thymidine kinase
TME	transmissible mink encephalopathy
TSEs	transmissible spongiform encephalopathies

i.c.	intracerebral(ly)
i.g.	intragastical(ly)
i.p.	intraperitonial(ly)
i.s.	intraspinal
i.v	intravenous(ly)
s.c.	subcutaneous(ly)

Abstract

To investigate the role of PrP in confering susceptibility to transmissible spongiform encephalopathies and furthermore to investigate the effect of restricted hamster PrP expression on the susceptibility to the hamster scrapie agent, we attempted to generate transgenic mice by using the hamster PrP open reading frame only, linked to appropriate regulatory 5' flanking sequences of brain specific genes. Low-level mRNA expression was achieved only in some of the transgenic lines generated and problems were encountered with PrP protein production. These data indicated that the promoter fragments used in the transgenic constructs did not contain all necessary elements to achieve high-level and specific expression and also that PrP translation was possibly regulated. It was postulated that other PrP sequences could be important for PrP mRNA regulation and efficient translation. The examination of the role of untranslated regions of the PrP mRNA was considered of major importance. The effect of 5' and 3' untranslated sequences of hamster PrP was tested on the translation of both PrP and lacZ. The effect of other regulatory sequences (a generic intron and an altered translation of initiation consensus sequence) was also investigated. However, none of the sequences tested appeared to have a significant effect on either PrP or lacZ translation efficiencies.

Contents

CHAPTER 1

Transmissible spongiform encephalopathies

Introduction

Transmissible spongiform encephalopathies (TSEs), comprise a complex set of neurodegenerative disorders, including kuru, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker syndrome (GSS) of humans, scrapie and bovine spongiform encephalopathy (BSE) of animals. These fatal diseases share prolonged incubation periods with progressive spongiform (vacuolar) degeneration of the cental nervous system (CNS) and variable amyloid plaque formation. TSEs are infectious diseases which can be transmitted to experimental animals. In addition, there are sporadic and genetic forms of TSEs.

Despite intensive investigations into the nature of the infectious agent, this issue remains one of the most intriguing in contemporary biology. Radiobiological and physicochemical data suggest that the infectious agent is different from conventional viruses and viroids. Although it appears to possess some viral properties, such as strain variation and mutation, there is no conclusive evidence that it contains any nucleic acid. On the contrary, the only characterised molecule that copurifies with infectivity is an abnormal isoform of a host encoded protein, designated cellular prion protein (PrP^{c}). During the course of the disease, PrP^{c} appears to undergo post-translational modification resulting in the infectious form, termed PrP^{sc}, which is partially resistant to proteinase K digestion.

Recent molecular genetic studies have provided evidence supporting a key role of the gene encoding PrP in the course of the disease. This gene is termed PRNP in human, Prn-p in mouse, but for simplicity the term PrP gene is used here to designate the gene encoding PrP protein irrespective of species.

Experiments with transgenic mice expressing PrP genes from various species have demonstrated that the PrP gene has an important

effect upon disease susceptibility, clinical-neuropathological features, incubation times and transmission between species.

In vitro studies involving expression of different PrP genes in cell lines and infection of tissue culture cells with the agent have provided models for studying certain aspects of TSEs.

The forms and main features of TSEs, as well as models developed to improve understanding of these diseases, are discussed in the following chapter.

1.1 Transmissible spongiform encephalopathies of man and other animals

The similarities in the clinical features and the neuropathological changes in the CNS, the long incubation periods leading to a protracted disease course and the species specificity of infection, were the main characteristics that led to the classification of six transmissible neurodegenerative diseases of animals and four of humans, as transmissible spongiform encephalopathies (Table 1). The association of PrP^{Sc} (proteinase K resistant PrP) with the pathogenesis of the disease and with neurodegeneration, and its accumulation in infected brains, have lately been recognised as the main features of TSEs.

Table 1. Transmissible spongiform encephalopathies

TSE	**host**
kuru	humans
Creutzfeldt-Jakob disease (CJD)	humans
Gerstmann-Sträussler-Scheinker syndrome (GSS)	humans
fatal familial insomnia (FFI)	humans
scrapie	sheep, goats
bovine spongiform encephalopathy (BSE)	cattle
transmissible mink encephalopathy (TME)	mink
chronic wasting disease (CWD)	mule deer, elk
feline spongiform encephalopathy (FSE)	cat, cheetah, puma
exotic ungulate encephalopathy	nyala, greater kudu oryx, gemsbok

1.2 TSEs : clinical features, transmissibility and epidemiology

1.2.1 TSEs of animals

1.2.1.1 Scrapie

Scrapie is a fatal ataxia of sheep and occasionally goats, that has been recognised for over two centuries and has a widespread distribution in Europe, Asia and America (Gajdusek, 1990). Affected animals show progressive ataxia, tremor, wasting and severe pruritis leading to wool and skin lesions (Sigurdsson, 1954).

The disease was first shown to be transmissible by inoculation of diseased brain tissue from sheep to a recipient ewe (Bensoit 1899, cited in (Gajdusek, 1990). In subsequent studies the transmissibility of scrapie to sheep was confirmed and the resemblance of the agent to viruses was demonstrated (cited in Gajdusek, 1990). When scrapie was first transmitted to mice, the neuropathological features appeared similar to those of sheep (Zlotnik and Rennie, 1962). Two different forms, or strains, of the disease were observed when scrapie was transmitted to goats (Pattison and Millson, 1961; Pattison and Millson, 1962): "scratching" and "drowsy", which were also transmitted to mice (Chandler, 1961). Transmission of the disease to hamsters produced a short incubation period model (Kimberlin and Walker, 1977). The passage of the infectious agent to laboratory animals led to the establishment of various scrapie models - since those initial experiments over 20 different strains of experimental scrapie have been characterised (Bruce and Fraser, 1991).

The way in which the disease spreads between sheep has not been established, although experimental infection of animals by oral route, scarification and via the conjunctiva point out possible natural routes (Kimberlin, 1990a). Evidence suggesting infection of lambs through the placenta (fetal membranes contain high amounts of infectivity), or from the dam after parturition, implied that maternal transmission might play a role in the natural spread of the disease (Kimberlin, 1990a).

1.2.1.2 Transmissible mink encephalopathy (TME)

TME is very similar to scrapie on the basis of pathological features and the physicochemical properties of the transmissible agent. First observed in 1947 in a Wisconsin mink herd (Hatsough and Burger, 1965), the disease has also been recognised in Canada and Finland (Gajdusek, 1990). Affected animals initially show subtle behavioural changes progressing to locomotor incoordination, ataxia, somnolence and, invariably, death. (Hatsough and Burger, 1965).

TME has been transmitted to several hosts, including monkey, sheep, goat and ferret (Gajdusek, 1990). Transmission to Syrian hamsters has also been established (Marsh and Kimberlin, 1975) and further studies using this experimental model revealed two different strains of the infectious agent: hyper (HY) and drowsy (DY) (Bessen and Marsh, 1992).

It was suggested that TME was caused by feeding of mink with cattle infected with an unrecognised scrapie-like agent (cited in Gajdusek, 1990). This view was supported by the finding that mink can be infected by oral dosing or intracerebral inoculation with sheep scrapie, producing a disease indistinguishable from scrapie (Hanson *et al.*, 1971). The high rate of horizontal spread of the disease was due to cannibalism and fighting between minks on the farms (Hatsough and Burger, 1965).

1.2.1.3 Chronic wasting disease (CWD) of mule deer and elk

Chronic wasting disease of mule deer was first described in a captive mule deer herd in a wildlife park in Colorado during 1967-1979 and later in Wyoming (Williams and Young, 1980). Disease signs closely resembled scrapie, but also included polydipsia, polyuria, excessive salivation and teeth grinding. Experimental transmission of the disease to other mule deer has been demonstrated (cited in Gajdusek, 1990). In the same wildlife park, a similar wasting disease occurred in captive Rocky Mountain elk (Williams and Young, 1982).

1.2.1.4 Bovine spongiform encephalopathy (BSE)

BSE, or "mad cow disease" was recognised retrospectively in 1985 in English dairy herds (Wells *et al.*, 1987). Predominant clinical features include hyperaesthesia, apprehension and hind-limb ataxia. The similarities of clinical, neuropathological (Wells *et al.*, 1987) and biochemical (Hope *et al.*, 1988) features of BSE and scrapie suggested that it was a novel TSE.

The disease has been experimentally transmitted to mice by a combination of intraperitoneal and intracerebral inoculation (Fraser *et al.*, 1988) and feeding with infectious brain homogenates (Barlow and Middleton, 1990). When other organs of the infected cattle were fed to mice, BSE transmission was not observed. The disease has also been experimentaly transmitted to pigs, cattle (Dawson *et al.*, 1990), marmosets (Baker *et al.*, 1993), sheep and goats (Foster *et al.*, 1993).

Epidemiological studies (Wilesmith *et al.*, 1988; Wilesmith *et al.*, 1991) suggest that the disease is not of genetic origin. It manifested as an epidemic caused by a common infectious source. The initial exposure of cattle to this source was estimated to be in the early 1980's (1981-1982). Incubation periods range from 2.5 to 8 years. No maternal, paternal or horizontal transmission of the disease has been observed (Bradley and Wilesmith, 1993). The most likely way that the disease spread to cattle was by the consumption of meat and bone meal supplement contaminated with sheep scrapie. The presence of infectivity in cattle feed was probably linked to changes in the rendering process. In particular, hydrocarbon solvent extraction from carcasses and steam treatment, part of the traditional extraction procedure, were discontinued during the early 1980's enabling the survival, or enrichment, of the scrapie agent (Wilesmith *et al.*, 1991; Bradley and Wilesmith, 1993). In that case, it has been hypothesised that the agent crossed the "species barrier" and created a novel disease in cattle. Other factors that could account for the BSE epidemic include an increase of the incidence of scrapie, the use of more sheep heads for rendering, the emergence of a new scrapie strain more pathogenic to cattle, or even a sporadic TSE of cattle. (Kimberlin, 1990a; Bradley and Wilesmith, 1993). Since the feed ban was introduced in 1988, ruminant protein was removed from cattle feed resulting in a decrease in the incidence of BSE

in younger animals (Wilesmith *et al.*, 1992). Measures were also taken in order to prevent the exposure of humans to BSE. The consumption of bovine offal was banned and it was recommended to destroy carcasses of suspect animals and their milk. Major issues concerning the human health risk, such as the amount of BSE-contaminated tissue that has already entered the human food chain and the frequency of BSE transmission to humans, still remain contentious. However, recent studies involving transgenic mice expressing the human PrP gene suggest that the BSE agent might not initiate the formation of human PrP^{Sc} in mice (see section 1.12.1).

1.2.1.5 Other TSEs of animals

Recently, TSE has been reported in members of the feline family including cat (Wyatt *et al.*, 1991), cheetah and puma (Willoughby *et al.*, 1992). Other captive exotic animals have been infected with the disease, such as nyala, eland, greater kudu, gemsbok and Arabian oryx. Transmission could have been through feed concentrates containing meat and bone-meal contaminated with scrapie or BSE (Wilesmith *et al.*, 1988), but in other cases in kudu, maternal transmission is also likely (Kirkwood *et al.*, 1993).

1.2.2 TSEs of man

1.2.2.1 Kuru

Kuru was described first in 1957 as an unusual progressive neurological disorder in the Fore tribe of Papua New Guinea (Gajdusek and Zigas, 1957). In the 1950s and 1960s it was the most common cause of death among the Fore population (approximately 35000) (Gajdusek, 1990). The disease (reviewed in Gajdusek, 1977; Gajdusek, 1990) is recognised by a progressive impairment of motor coordination with dysarthria, shivering-like tremor, cerebellar ataxia, and often dementia in the terminal stages. Victims would usually die within 2 years of onset after long incubation periods of approximately 4-30 years. The similarity

between kuru and scrapie, based on neuropathological features, was first pointed out in 1959 (Hadlow, 1959).

This link was strengthened when kuru was demonstrated to be transmissible to chimpanzees (Gajdusek *et al.*, 1966). Later, the disease was transmitted to New World and Old World monkeys and to mink and ferret. Many other nonprimate species, including laboratory rodents, have remained resistant to inoculation with kuru infectious agent (Gajdusek, 1990).

Epidemiological studies (Gajdusek, 1977; Gajdusek, 1990) have revealed that the spread of kuru was connected to the endocannibalism of dead relatives, a rite of mourning among the Fore tribe. This ritual was performed by females and their young children. Apart from the oral route, infection via intradermal (damaged skin), nasal and ocular routes could have occurred. Males over 6 years old did not usually handle or eat dead kinsmen, accounting for the high incidence of disease among women and the distinct age distribution. The incidence of Kuru has declined since the cessation of ritualistic cannibalism in 1956 and the disease no longer appears in children or young adults.

1.2.2.2 Creutzfeldt-Jakob disease (CJD)

CJD presents as a progressive dementing illness of man, first reported by Creutzfeldt in 1920 and Jakob in 1921 (cited in Bell and Ironside, 1993). It was not until 1959 that its resemblance to scrapie was noted (Klatzo *et al.*, 1959) and 1979 that a more thorough analysis of the disease was presented (Masters *et al.*, 1979). The onset of the disease is usually during the sixth decade of life (Will and Matthews, 1984) and the course between one and thirty-five months (Kretzschmar, 1993). Clinical signs can be variable (Brown *et al.*, 1993). Typical features include initial memory loss and diminished intellect, followed by rapidly progressive dementia leading to death. Other cases present as cerebellar syndromes with diminished coordination, myoclonous, tremor and ataxia, that eventually result in dementia.

The disease has been successfully transmitted to chimpanzees and New and Old World monkeys. Only some of the CJD isolates can be

transmitted to cat, guinea pig, hamster and mouse (cited in Gajdusek, 1990).

CJD occurs mainly as a sporadic illness, with no correlation between the incidence of the disease (1 case per million per annum) and any possible source of infection (Masters *et al.*, 1981a; Brown *et al.*, 1987). Approximately 5-15% of CJD cases are familial, showing an autosomal dominant pattern of disease segregation (Will, 1993; Masters *et al.*, 1979). A very small proportion of disease incidence is due to iatrogenic transmission, which depends on direct exposure of the recipient with contaminated material. These cases have been reported to arise through contaminated neurosurgical instruments, depth electrodes, corneal and dura mater grafts, pituitary derived gonadotropin and growth hormone from affected donors (Will, 1993; Brown *et al.*, 1992; Smith and Collinge, 1995).

1.2.2.3 Gerstmann-Sträussler-Scheinker syndrome (GSS)

GSS was described in 1928 as a slowly progressive ataxia. It is now regarded as a familial disorder with an autosomal dominant pattern of inheritance, clinically characterised by lack of coordination, speech difficulties, limb weakness and chronic cerebellar ataxia with dementia occurring later in the course of the disease. Typically the disease manifests at the fifth decade of life, with duration ranging between 1 and twenty years. The incidence of GSS is very low : 2 cases per 100 million per year (Prusiner, 1993a). It was succesfully transmitted to primates in 1981 (Masters *et al.*, 1981a).

1.2.2.4 Fatal familial insomnia (FFI)

Recently FFI was described as another familial TSE disease (Medori *et al.*, 1992) with an autosomal dominant pattern of disease segregation. Clinical signs include agitation during sleep and progressive insomnia, dysarthria, ataxia and memory impairment. The disease usually manifests at 40-60 years of age and the duration varies between 7-18

months (reviewed in Gambetti *et al.*, 1993). FFI has been recently transmitted to mice (Tateishi *et al., 1995).*

1.3 Diversity in neuropathology of TSEs

One of the most important criteria by which these diseases are classified is the pathology of the CNS (reviewed in Bell and Ironside, 1993; Fraser, 1993; Fraser *et al.*, 1986). Generally it consists of non-inflammatory, degenerative vacuolation of neurons and spongiform change of the neuropil, usually confined to the grey matter. The disease progresses with neuronal loss, gliosis and hypertrophy of astrocytes. Formation of cerebral plaques due to extracellular deposits of amyloid are an inconsistent feature, related to some forms of TSEs. The pathology is influenced by the genetic differences of the host and variations in the origin of the infectious agent. Aggregates of PrP^{Sc} purified from diseased brain homogenates, designated "scrapie-associated fibrils" (SAF) (Merz *et al.*, 1981), are usually part of the molecular pathology of the disease and regardless of their significance, their presence has been useful for confirming TSE cases (Gibbs *et al.*, 1985; Hope *et al.*, 1988). The detection of PrP^{Sc}, the protease-resistant, disease-associated isoform of PrP^{C}, which is thought to have a central role in the etiology and pathogenesis of the disease, can also serve as a diagnostic marker. Assays developed to detect PrP^{Sc} and distinguish between PrP^{Sc} and PrP^{C} could result in providing a more reliable diagnostic method (Tateishi and Kitamoto, 1993, reviewed in Bell and Ironside, 1993).

In neurons, PrP^{Sc} has been detected in synaptic structures (Kitamoto *et al.*, 1991), dendrites and terminal axons (DeArmond *et al.*, 1987), in contrast to PrP^{C} which is detected in the cell bodies (Kitamoto *et al.*, 1991). PrP^{Sc} usually accumulates on the plasmalemma of neurons, diffusing from the cell surface to extraneuronal space and dense accumulation of PrP^{Sc} is associated with disruption of the neuropil and vacuolation (Jeffrey *et al.*, 1994a; Jeffrey *et al.*, 1994b). PrP^{Sc} can also be detected in astrocytes (Diedrich *et al.*, 1991). The site of PrP^{Sc} accumulation in astrocytes and microglial cells is thought to be the lysosomes (Jeffrey *et al.*, 1994c).

In naturally occurring TSEs of animals, neuropathology appears generally similar (reviewed in Fraser, 1993; Fraser *et al.*, 1986). Most prominent features are the vacuolation of neurons in the spinal cord, medulla, pons and midbrain, while the cerebrum is usually unaffected. Spongiform degeneration in the neuropil occurs in the brain stem, septum and hypothalamus (Zlotnik, 1958) and pathology is accompanied by astrocytic hypertrophy. Some animals affected with TME, CWD and FSE show cerebral cortical spongiform lesions and in a few cases of scrapie and CWD severe cerebrovascular amyloidosis has been observed (Willoughby *et al.*, 1992; Williams and Young, 1980; Wyatt *et al.*, 1991; Hatsough and Burger, 1965; Wood and Done, 1992).

Human TSEs manifest with a wide range of neuropathology (reviewed in Bell and Ironside, 1993; Lantos, 1992). Spongiform change in the cerebral cortex, grey matter and cerebellum, accompanied by proliferation and hypertrophy of the astrocytes, is evident in all kuru cases, but the most striking feature is the presence of amyloid plaques, mainly in the cerebellum, in approximately 70% of the cases (Gajdusek, 1990; Bell and Ironside, 1993).

In most CJD cases cortical atrophy has been described and in a few cases cerebellar atrophy is also prominent (Bell and Ironside, 1993). Characteristic histological findings include spongiform degeneration of the neuropil, neuronal loss, astrocytosis and sometimes amyloid plaque formation. Additionally, spongiform change in the cerebral white matter is observed in some cases. However, these neuropathological changes are not consistent and the severity and distribution of lesions differ substantially from case to case (Bell and Ironside, 1993; Lantos, 1992).

Histopathology in GSS cases is dominated by accumulation of amyloid plaques throughout the cerebrum and cerebellum, with spongiform change in the cerebral cortex occurring only in some cases (Bell and Ironside, 1993; Masters *et al.*, 1981a).

The most prominent feature of FFI is thalamic atrophy. Neuronal loss, gliosis and spongiosis have also been observed in the thalamus, cerebral cortex, and cerebellum (Gambetti *et al.*, 1993).

1.4 The nature of the infectious agent

Contradictory views have been presented concerning the nature of the TSE infectious agent. Although most scientific evidence to date argues against the requirement for a conventional virus or viroid, the possibility of a nucleic acid component cannot be excluded. Early studies demonstrated that infectivity was resistant to heating, treatment with acetylethyleneimine, ultra-violet and ionizing radiation (Stamp *et al.*, 1959; Alper *et al.*, 1966; Alper *et al.*, 1967). Further investigations showed that the agent was resistant to treatment with DNase, RNase, formalin, ether, pepsin and high salt concentrations (Pattison and Millson, 1961; Pattison and Millson, 1960; Mould and Smith, 1995). These findings were confirmed and extended when a range of procedures that inactivate, degrade, or modify nucleic acids appeared to have little effect on infectivity (Gajdusek, 1977; BellingerKawahara *et al.*, 1987a; BellingerKawahara *et al.*, 1987b). Although the possibility of a nucleic acid being part of the agent was not completely eliminated, it would have to be quite small and well protected. Other researchers have questioned the methodology used to produce these results and have presented evidence suggesting that the infectious agent resembles a virus both in size and in susceptibility to inactivating procedures (Rohwer, 1984).

Subsequent physicochemical studies suggested that this putative nucleic acid would have to be very small (approximately 100 bp or less), very efficient (low molecule to infectivity ratio) and heterogeneous in size (Meyer *et al.*, 1991; Kellings *et al.*, 1992). In contrast, other investigators have been able to identify nucleic acids (500-6000 bp length) in infectious preparations (Sklaviadis *et al.*, 1990; Akowitz *et al.*, 1990), which appeared to be similar to nuclease protected viral cores (Sklaviadis *et al.*, 1990; Akowitz *et al.*, 1990). A small single-stranded D-loop mitochondrial DNA fragment has been shown to be enriched in highly infectious preparations (Aiken *et al.*, 1990; Aiken and Marsh, 1990) and retroviral-like sequences present in these preparations have been cloned (Murdoch *et al.*, 1990; Akowitz *et al.*, 1993). Also, small virus-like particles (Ozel and Diringer, 1994), single-stranded DNA associated with proteins (Narang *et al.*, 1988; Narang, 1990) and multimeric mitochondrial DNA (Narang, 1990) have been visualised by

electron microscopy in highly infectious fractions. The contribution of any of these molecules to the infectious agent has not yet been established. However, subtractive hybridisation and cloning methodologies have failed to identify a nucleic acid (Aiken and Marsh, 1990; Duguid and Dinauer, 1990; Duguid *et al.*, 1989; Duguid *et al.*, 1988; Diedrich *et al.*, 1987), but the sensitivity of these techniques has been questioned (Aiken and Marsh, 1990).

The unusual properties of the agent and the identification of a hydrophobic protein that copurified with infectivity (Prusiner, 1982) led to the proposal of the term 'prion' (Prusiner, 1982) in order to distinguish the particles from viruses and viroids. Although this protein, designated PrP, is now generally accepted to be the main component of the agent, there are contradictory views on its association with infectivity (Sklaviadis *et al.*, 1990; Manuelidis *et al.*, 1987; Bolton *et al.*, 1982; Prusiner *et al.*, 1982). Different hypotheses on the nature and replication of the agent will be further discussed in (1.13).

1.5 PrP gene

Partially purified infectious fractions demonstrated that the infectious agent was likely to be hydrophobic, had a tendency to aggregate and was sensitive to phenol, chaotropic ions and denaturing detergents, suggesting the possibility of a protein component (Prusiner *et al.*, 1980a; Prusiner *et al.*, 1978). Further purification revealed the existence of a hydrophobic protein, designated PrP 27-30, which was partially resistant to proteinase K digestion and had molecular weight of 27-30 kD (Prusiner *et al.*, 1981; Prusiner *et al.*, 1980b; Prusiner *et al.*, 1982; McKinley *et al.*, 1983). Once the sequence of PrP 27-30 was determined (Prusiner *et al.*, 1984), oligonucleotides corresponding to that sequence were synthesised and used to identify a PrP cDNA (Oesch *et al.*, 1985; Chesebro *et al.*, 1985). Southern blotting with PrP cDNA revealed a single-copy chromosomal gene that gave the same restriction patterns when infected and normal brain samples were compared (Basler *et al.*, 1986).

Since the isolation of hamster PrP sequences (Oesch *et al.*, 1985; Basler *et al.*, 1986; Chesebro *et al.*, 1985; Robakis *et al.*, 1986; Lowenstein *et*

al., 1990), PrP genes from other species, including human (Puckett *et al.*, 1991; Liao *et al.*, 1986; Kretzschmar *et al.*, 1986b), mouse (Locht *et al.*, 1986; Westaway *et al.*, 1987), sheep (Goldmann *et al.*, 1990), cattle (Goldmann *et al.*, 1991b) and mink (Kretzschmar *et al.*, 1992) have been characterised. The PrP gene has also been cloned from a bird (chicken) (Gabriel *et al.*, 1992; Harris *et al.*, 1991) and a marsupial (possum) (Windl *et al.*, 1995). PrP-related sequences have been identified in Drosophila melanogaster, Caenorhabditis elegans and yeast (Westaway and Prusiner, 1986), but attempts to prove the existence of a PrP-like protein in these species have failed. Attempts to clone the C. elegans PrP gene that hybridised with the hamster PrP cDNA resulted in the identification of an unrelated gene (a novel heterologous nuclear ribonucleoprotein gene) (Iwasaki *et al.*, 1992). These results suggest that the PrP gene is confined to mammals and some other vertebrates. The conservation of the open reading frame (ORF) between mammals is over 81% at the nucleic acid level and over 85% at the amino acid level (Kretzschmar *et al.*, 1992; Goldmann, 1993). The similarity of the amino acid sequence between mouse and chicken is 33% (Laplanche *et al.*, 1993). PrP shows almost no homology to other known proteins (Goldmann, 1993).

The human PrP gene (PRNP) was shown to be located on chromosome 20 (Puckett *et al.*, 1991; Liao *et al.*, 1986; Sparkes *et al.*, 1986) and the mouse PrP gene (Prn-p) on chromosome 2 (Sparkes *et al.*, 1986). The PrP ORF is contained within a single exon, eliminating the possibility of alternative splicing, and this exon is separated from an upstream PrP exon by a 10 kb intron (Basler *et al.*, 1986; Puckett *et al.*, 1991). The PrP ORF has a similar length (approximately 760 bp) in all mammals. Examples of the PrP gene and mRNA structure are given in Figure 1.1.

The promoter of the PrP genes has not been well defined. Sequence data have revealed that there is no typical consensus TATA box for transcription initiation. A prominent feature is a high content of G and C nucleotides, particularly around the transcription start site (Puckett *et al.*, 1991; Westaway *et al.*, 1987; Basler *et al.*, 1986). The promoter of the PrP gene contains consensus sequences for the binding of ubiquitous transcription factors such as Sp1 and AP-1 and hence resembles housekeeping gene promoters.

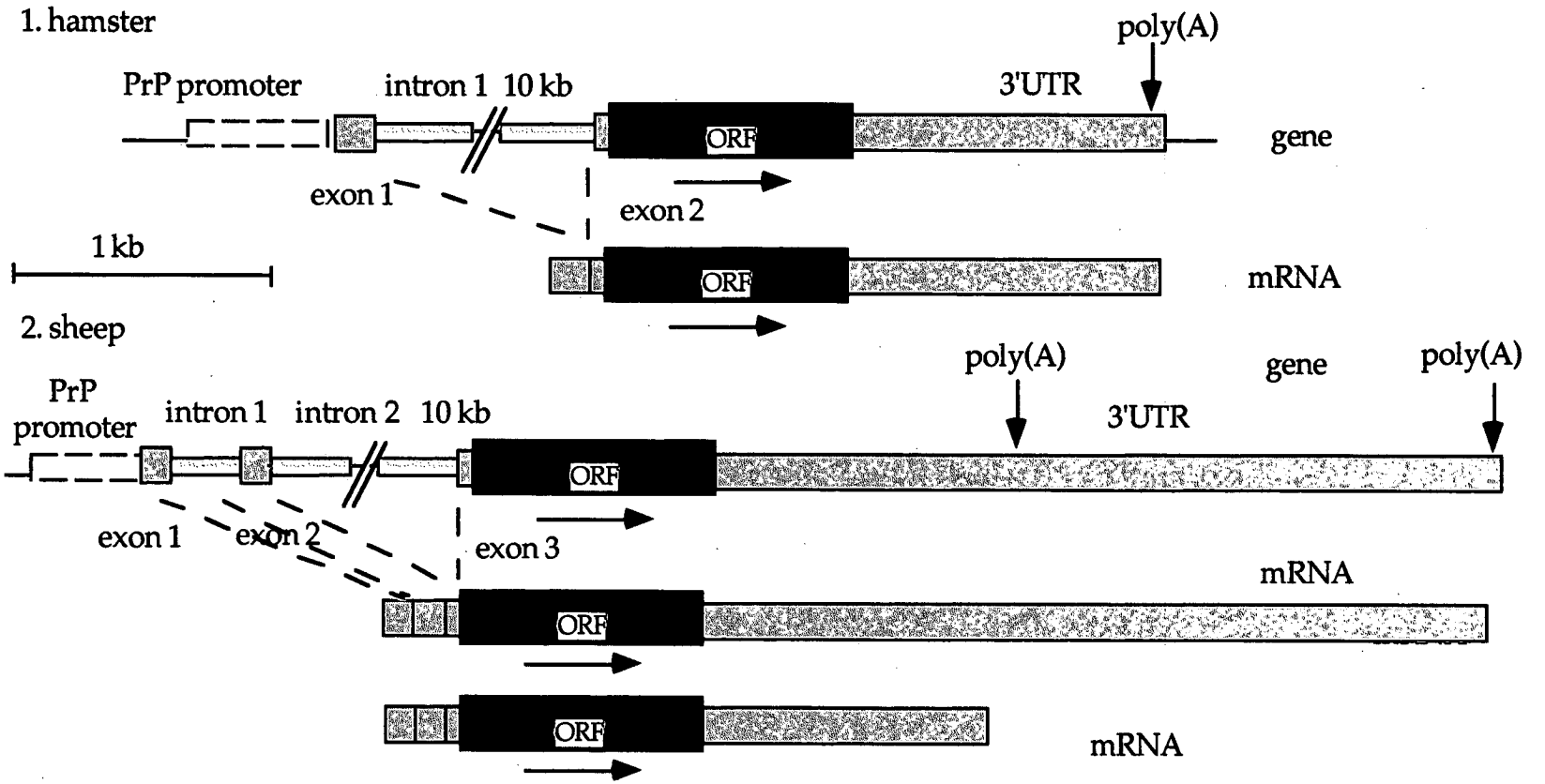


Figure 1.1 Structure of PrP gene and transcription products

1. The hamster PrP gene consisting of two exons and the hamster PrP mRNA.

2. The sheep PrP gene consisting of 3 exons. Two different size transcripts are probably a result of the use of an additional poly(A) addition site (Goldmann, 1993).

The 5' leader sequence of the PrP transcript is encoded by one exon in hamster and man and by two exons in mouse and sheep (Puckett *et al.*, 1991; Basler *et al.*, 1986; Westaway *et al.*, 1987; Goldmann, 1993). Differences in the length of the 5' leader sequence have been observed in mouse and hamster PrP mRNA, due to multiple transcription initiation sites while, in human, only one major initiation site has been detected (Puckett *et al.*, 1991; Basler *et al.*, 1986; Westaway *et al.*, 1987).

A conserved feature of the PrP ORF is that it contains a GC rich region in the 5' half, organised usually in 24 bp repeats coding for almost identical octapeptides (Oesch *et al.*, 1985; Liao *et al.*, 1986; Goldmann *et al.*, 1990; Goldmann *et al.*, 1991b; Locht *et al.*, 1986; Kretzschmar *et al.*, 1992).

The 3' untranslated region (3' UTR) of PrP mRNA is quite long and conserved between humans, rodents and sheep, apart from two sheep-specific insertions (Goldmann *et al.*, 1990). In particular, there is a AT-rich domain upstream of the poly(A) addition signal and near the end of the mRNA, that is highly homologous between species (Goldmann, 1993; Goldmann *et al.*, 1990). Two mRNA transcripts are present in sheep, thought to be produced through alternative use of poly(A) addition signals in the sheep 3' UTR (Hunter *et al.*, 1994; Horiuchi *et al.*, 1995).

1.6 PrP expression

PrP mRNA in mouse is detected mainly in the brain and the highest expression was shown to be in the brainstem, hippocampus, thalamus, cortex and cerebellum (Manson *et al.*, 1992a; Mobley *et al.*, 1988). *In situ* hybridisation has revealed that PrP is expressed in mouse embryos after 13.5 days throughout the developing neural tube and after 16 days in non-neuronal cells, with high levels observed in specific kidney cells and tooth buds (Manson *et al.*, 1992b). In neonatal mice PrP mRNA has been shown to be developmentally regulated; it increases at different rates in various brain regions (McKinley *et al.*, 1987). During the first 20 days after birth PrP mRNA increases four-fold and remains at that level throughout adult life (Lazarini *et al.*, 1991). It continues to be

expressed at high levels in most, if not all, neurons in the adult brain, as well as in ganglia and peripheral nerves (Kretzschmar *et al.*, 1986a; Manson *et al.*, 1992b; Manson *et al.*, 1992a). Expression in many non-neuronal cells, including astrocytes, pericytes and ependymal, epithelial and endothelial cells, has also been reported (Brown *et al.*, 1990). PrP mRNA has been detected in smaller amounts in many other tissues tested, such as heart, lung, kidney, intestine, skeletal muscle, spleen, adrenal gland, lymph node, ovary and testis, but not in liver (Chesebro *et al.*, 1985; Horiuchi *et al.*, 1995; Caughey *et al.*, 1988a; Bendheim *et al.*, 1992; Oesch *et al.*, 1985; Brown *et al.*, 1990; Robakis *et al.*, 1986). It has been observed that PrP mRNA expression can be induced by nerve growth factor (NGF) (Mobley *et al.*, 1988). There is no change in the level, size, or localisation of PrP mRNA during the course of the disease (Chesebro *et al.*, 1985; Manson *et al.*, 1992a).

The human PrP mRNA is 2.7 kb, the bovine is 4.5 kb (same as in sheep and goats), the hamster 2.1 kb and the mouse 2.3 kb.

1.7 PrP protein

1.7.1 Primary structure and post-translational modifications of PrP

PrP^{27-30}, which was identified as the most abundant component in the infectious preparations (Prusiner *et al.*, 1982; Prusiner *et al.*, 1984), is an isoform of a normal cellular protein, PrP^{C}, encoded by the PrP gene of the host (Oesch *et al.*, 1985; Basler *et al.*, 1986). When PrP^{C} was isolated from normal brain it appeared as a soluble, protease sensitive protein of molecular weight 33-35 kD (Oesch *et al.*, 1985; Meyer *et al.*, 1986; Barry *et al.*, 1986; Bendheim *et al.*, 1988). In the purified infectious preparations, the disease-associated form, PrP^{Sc}, is insoluble after detergent extraction and tends to aggregate (Oesch *et al.*, 1985; Prusiner *et al.*, 1981). It shows, though, the same molecular weight as PrP^{C}, 33-35 kD, which is reduced to 27-30 kD (PrP^{27-30}) after proteinase K digestion (Prusiner *et al.*, 1984; Hope et al,. 1986; Turk *et al.*, 1988; Prusiner *et al.*, 1982). This partial proteinase K resistance is a feature widely used for diagnosis of TSEs.

The primary structure of PrP^{Sc} has been shown to be identical to that predicted for PrP^{C}, by amino acid sequencing and mass spectrometry

(Stahl *et al.*, 1993; Baldwin *et al.*, 1993). The charge and size heterogeneity observed with electrophoretic separation techniques indicates extensive post-translational modifications for both isoforms (Meyer *et al.*, 1986; Hope *et al.*, 1986).

Amino acid sequences of the PrP protein from different species have been deduced from genomic and cDNA sequences and some have been confirmed by protein sequencing (Puckett *et al.*, 1991; Basler *et al.*, 1986; Locht *et al.*, 1986; Goldmann *et al.*, 1990; Goldmann *et al.*, 1991b; Kretzschmar *et al.*, 1992; Prusiner *et al.*, 1984; Hope *et al.*, 1986; Turk *et al.*, 1988). PrP proteins of different species exhibit above 85% similarity and the structural characteristics are conserved. The length of the predicted precursor protein is 253 amino acids for human PrP, 254 for hamster and mouse, 257 for mink and 256 for sheep. The structure of the PrP protein (hamster) is presented in Figure 1.2.

A series of studies (Hope *et al.*, 1986; Basler *et al.*, 1986; Goldmann *et al.*, 1990; Puckett *et al.*, 1991; Kretzschmar *et al.*, 1992; Prusiner *et al.*, 1984; Turk *et al.*, 1988; Locht *et al.*, 1986; Bazan *et al.*, 1987; Goldmann *et al.*, 1991b) have shown that the amino-terminal 22 amino acids (24, in sheep, cattle, mink) form the signal peptide, which is cleaved off after guiding the protein into the endoplasmic reticulum. PrP of all species contains 2 hexapeptide repeats and usually 5 glycine-rich octapeptide repeats (codons 51 to 90). In cattle, the most common allele contains 6 octarepeats (Goldmann *et al.*, 1991b). Two further tandem repeats (codons 148 to 159 and 161 to 171) and an overlapping aromatic palindrome (codons 147 to 163) (Sulkowski, 1992) are also present in the PrP sequence. Although these repeats appear to be unique to PrP, the core of the octapeptide repeats is similar to motifs found in keratin (Oesch *et al.*, 1985). No function has been assigned to these motifs. However, it has been postulated that the palindromic sequences could play a role in dimerisation of PrP, or binding of other proteins to PrP (Goldmann, 1993). In man, insertions of integral numbers of the octapeptide repeats are linked to inherited TSEs (discussed in section 1.8), but data from experiments using cell culture systems have indicated that the deletion of that region or the addition of extra octarepeats does not influence the conversion of PrP^{C} to PrP^{Sc} (Rogers *et al.*, 1993). Also, it was recently demonstrated that introduction of PrP transgenes carrying

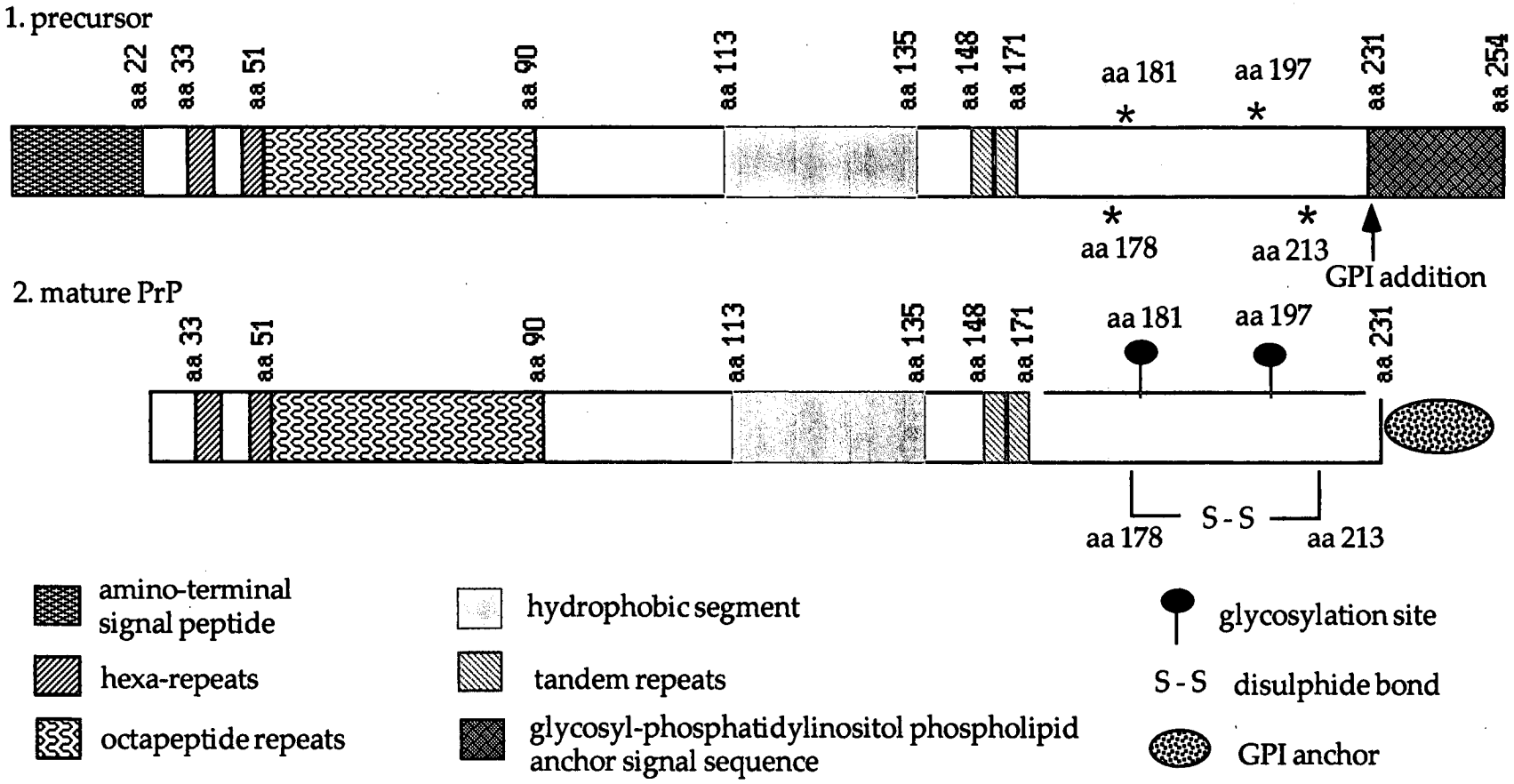


Figure 1.2 Structural features of PrP protein (hamster)

1. Precursor PrP protein.
2. Post-translationally modified PrP protein

octapeptide repeat deletions into PrP null mice conferred susceptibility to the disease, but the levels of PrP^{Sc} were much lower than those observed in infected wild type mice (Fischer *et al.*, 1996).

A hydrophobic, transmembrane α-helix domain (codons 113 to 135) and amphipathic α-helix (codons 157 to 177) were proposed to cross the lipid bilayer. This idea was supported by secondary structure predictions through computational analysis (Bazan *et al.*, 1987) and by *in vitro* translation studies (Yost *et al.*, 1990; Lopez *et al.*, 1990; Hay *et al.*, 1987a). However, it is now well established that PrP is attached to the cell membrane by a glycosyl-phosphatidylinositol phospholipid (GPI) anchor and can be detached by enzymatic digestion with phosphatidylinositol-specific phospholipase C (PIPLC) (Stahl *et al.*, 1987; Stahl *et al.*, 1990c; Stahl *et al.*, 1990a). The GPI anchor attachment signal, formed by the hydrophobic carboxy-terminal 23 amino acids (codons 232 to 254), is removed prior to the GPI attachment. The site of the anchor attachment is serine 231 (Stahl *et al.*, 1990b; Stahl *et al.*, 1990a). The structure of the GPI moiety appears to be heterogeneous (2-2.5 kD), with 30% of the carbohydrate structure consisting of sialic acid, but no differences have been observed between the GPI anchors of PrP^{C} and PrP^{Sc} (Stahl *et al.*, 1992; Stahl *et al.*, 1990a). Furthermore, cell culture experiments have demonstrated that the presence of the GPI anchor is not required for the conversion of PrP^{C} to PrP^{Sc} (Rogers *et al.*, 1993; Kocisko *et al.*, 1994). However, when a C-terminal truncated (including the GPI addition site) PrP transgene was introduced into PrP null mice no PrP protein was produced, suggesting that the protein may have been secreted and degraded rapidly (Fischer *et al.*, 1996). A secreted form of PrP has been reported in cerebrospinal fluid (Tagliavini *et al.*, 1992), in cell-free translation systems (Lopez *et al.*, 1990; Yost *et al.*, 1990) and Xenopus oocytes (Hay *et al.*, 1987b), but its significance is not known.

Two cysteine residues (codons 178 and 213) are linked by an intramolecular disulfide bond, which creates a loop including two asparagine-linked glycosylation sites (codons 181 and 197) (Turk *et al.*, 1988; Bazan *et al.*, 1987). However, PrP is not always glycosylated at these sites; the existence of unglycosylated, monoglycosylated and diglycosylated isoforms has been confirmed directly (Somerville and Ritchie, 1990) and by cell culture experiments (Caughey *et al.*, 1989). The

size heterogeneity of the unglycosylated form (observed on SDS-PAGE electrophoresis), is due to variations in the GPI anchor and *in vivo* proteolysis (Stahl *et al.*, 1992; Somerville and Ritchie, 1990; Hope *et al.*, 1988c; Hope and Hunter, 1988). Structural analysis of the asparagine-linked sugar moieties performed on PrP^{Sc} demonstrated over 400 different oligosaccharide combinations (Endo *et al.*, 1989). Whether this is true for PrP^{C} and whether it is important for the formation and diversity (strains) of the infectious agent remain to be established. However, it has been demonstrated, that the conversion of PrP^{C} to PrP^{Sc} is still possible when glycosylation is inhibited (Rogers *et al.*, 1990).

Another modification that occurs in both PrP^{C} and PrP^{Sc} is the conversion of two arginine residues (codons 25 and 37) to citrulline (Hope, 1993). Although PrP has 2 threonine phosphorylation sites (codons 183 and 192), a tyrosine phosphorylation site (codon 155, not present in human PrP) and a tyrosine sulphation motif (codon 145, not present in rodent PrP) their utilisation has not been experimentally demonstrated (Goldmann, 1993).

These data suggest that there are no obvious covalent differences between PrP^{C} and PrP^{Sc}, although an amino acid substitution or a post translational modification occurring in a very small fraction of PrP^{Sc} could be covered by the general heterogeneity observed, or could have escaped the detection methods.

1.7.2 Secondary structure and conformation of PrP

The secondary structure of PrP^{C} is predominantly α-helical, (42%) with little content in ß-sheet (3%) (Pan *et al.*, 1993). In contrast the ß-sheet content of PrP^{Sc} is high (34% ß-sheet, 20% α-helix, 46% ß-turns and random coil). Characterisation of PrP^{27-30} also showed a high content in ß-sheet (43% ß-sheet, no α-helix, 57% ß-turns and random coil) (Safar *et al.*, 1993). Furthermore, treatments that were found to reduce infectivity, such as high pH and SDS denaturation, also reduced the ß-sheet content of PrP^{27-30} (Gasset *et al.*, 1993). This finding is not surprising since it is known that amyloids generally contain proteins with ß-sheet conformation. Whether this conformational change is related to the pathogenesis of the disease still remains to be established.

It was proposed that PrP has four regions of α-helices. When these were synthesised as peptides, three of them formed a ß-sheet conformation *in vitro* (Gasset *et al.*, 1992). These peptides were also shown to be neurotoxic (Forloni *et al.*, 1993). It was therefore hypothesised that PrP has a three-dimensional conformation, consisting of four regions of α-helices (Huang *et al.*, 1994) and some of them could undergo a conversion into ß-sheets spontaneously, or through mutations in the PrP primary sequence, or triggered by the interaction with the infectious agent (Goldfarb *et al.*, 1993; Huang *et al.*, 1994; Sulkowski, 1992).

1.8 Genetics of TSEs

Following experimental transmission of scrapie to mice (Chandler, 1961) a series of studies involving different inbred mouse strains produced evidence supporting the influence of a single autosomal gene, designated Sinc, on scrapie incubation periods (Dickinson *et al.*, 1968). Further characterisation of this gene led to the suggestion that there were two alleles in mice, termed $Sinc^{s7}$ and $Sinc^{p7}$, that controlled the incubation periods after infection with the different scrapie-agent strains (Dickinson and Meikle, 1971).

Studies on the Prn-p genes (PrP genes) of mice that exhibited short and long incubation periods, demonstrated a genetic linkage between a gene thought to modulate the incubation periods, designated Prn-i, and a restriction fragment length polymorphism of Prn-p (Carlson *et al.*, 1986; Carlson, 1991). Following the cloning of the mouse PrP gene, two alleles were identified, Prn-p^a and Prn-p^b, which differed by two amino acids, at codon 108: Leu or Phe and at codon 189: Thr or Ala, respectively (Locht *et al.*, 1986; Westaway *et al.*, 1987). It was demonstrated that Prn-p^a segregated with the $Sinc^{s7}$ and Prn-p^b with the $Sinc^{p7}$ allele (Carlson *et al.*, 1986; Carlson *et al.*, 1988; Carlson *et al.*, 1991).

Other researchers have confirmed the linkage between Prn-p and Prn-i/Sinc, but it has not been unequivocally proven that they are congruent (Carlson *et al.*, 1986; Carlson *et al.*, 1988; Hunter *et al.*, 1992).

The incubation period gene for experimental scrapie in Cheviot sheep, termed Sip, was also shown to be linked to PrP gene restriction

fragment length polymorphisms (Hunter *et al.*, 1989). It was subsequently demonstrated that four PrP protein variants were associated with the short (sA) and prolonged (pA) incubation period alleles (Goldmann *et al.*, 1991a). Further studies with different strains of agent supported the correlation between dimorphisms in codons 136 and 171 of the ovine PrP gene and the modulation of the incubation period and disease incidence (Goldmann *et al.*, 1994; Westaway *et al.*, 1994b).

In humans, a genetic contribution was recognised when approximately 10% of CJD cases were found to be familial (Masters *et al.*, 1981b). Genetic linkage studies in mice and the discovery of the human PrP gene (see section 1.5) led to a search for mutations that might feature in human hereditary disease. Since a point mutation at codon 102 (Pro ->Leu) was shown to be linked to GSS, many other pathogenic mutations and non-pathogenic polymorphisms have been identified (Prusiner, 1994b; Prusiner and DeArmond, 1994; Smith and Collinge, 1995; Prusiner and Hsiao, 1994; Gajdusek, 1993; Prusiner, 1994a), summarised in Table 2 and Figure 1.3. The polymorphism at codon 129 can determine the phenotype of the disease when the 178 Asp-> Asn mutation is present. The 129 Met 178 Asn combination was shown to segregate with FFI, whereas 129 Val 178 Asn segregated with CJD. Studies involving many normal individuals and sporadic CJD cases have revealed that homozygosity at the 129 codon predisposes to the disease (Palmer *et al.*, 1991; De Silva *et al.*, 1994; Windl *et al.*, submitted). Other researchers have reported that 129 Val influences the pathological features of sporadic CJD (Miyazono *et al.*, 1992) and in familial GSS 198 Phe->Ser, 129 Val homozygosity is responsible for early onset of the disease (Dlouhy *et al.*, 1992).

The availability of these genetic markers has facilitated the study of the remarkably heterogeneous phenotypes of inherited TSEs and the recognition of cases that were not considered previously to be TSE.

1.9 Pathogenesis of TSEs

Because the infectious agent has not been unequivocally identified, the spread and the dynamics of infectivity have been studied only with

Table 2. Pathogenic mutations and non-pathogenic polymorphisms of the human PrP gene.

pathogenic mutations	disease	references
octapeptide repeat insertions	familial CJD	Owen *et al.*, 1990; Owen *et al.*, 1991; Goldfarb *et al.*, 1991a; Goldfarb *et al.*, 1993
aa 102 Pro->Leu	familial GSS	Hsiao *et al.*, 1989; Kretzschmar *et al.*, 1991
aa 105 Pro->Leu	familial GSS	Kitamoto *et al.*, 1993b
aa 117 Ala->Val	familial GSS	DohUra *et al.*, 1989
aa 145 Tyr->stop	familial GSS	Kitamoto *et al.*, 1993a
aa 178 Asp->Asn 129 Val	familial CJD	Goldfarb *et al.*, 1991c
aa 178 Asp->Asn 129 Met	familial FFI	Goldfarb *et al.*, 1992; Medori *et al.*, 1992; Gambetti *et al.*, 1993
aa 180 Val->Iso	sporadic CJD	Hitoshi *et al.*, 1993; Tateishi and Kitamoto, 1995
aa 198 Phe->Ser	familial GSS	Dlouhy *et al.*, 1992; Tagliavini *et al.*, 1994; Hsiao *et al.*, 1992; Tagliavini *et al.*, 1991
aa 200 Glu->Lys	familial CJD	Goldfarb *et al.*, 1990; Goldfarb *et al.*, 1991b; Bertoni *et al.*, 1992
aa 210 Val->Iso	familial CJD	Pocchiari *et al.*, 1993; Ripoll *et al.*, 1993
aa 217 Gln->Arg	familial GSS	Hsiao *et al.*, 1992; Tagliavini *et al.*, 1994
aa 232 Met->Arg	sporadic CJD	Hitoshi *et al.*, 1993; Tateishi and Kitamoto, 1995

non-pathogenic polymorphisms		
aa 117 (no aa change)		Gajdusek, 1993
aa 129 Met->Val	see text	Palmer *et al.*, 1991; De Silva *et al.*, 1994
octapeptide repeat deletion		Palmer *et al.*, 1993; Pocchiari *et al.*, 1993

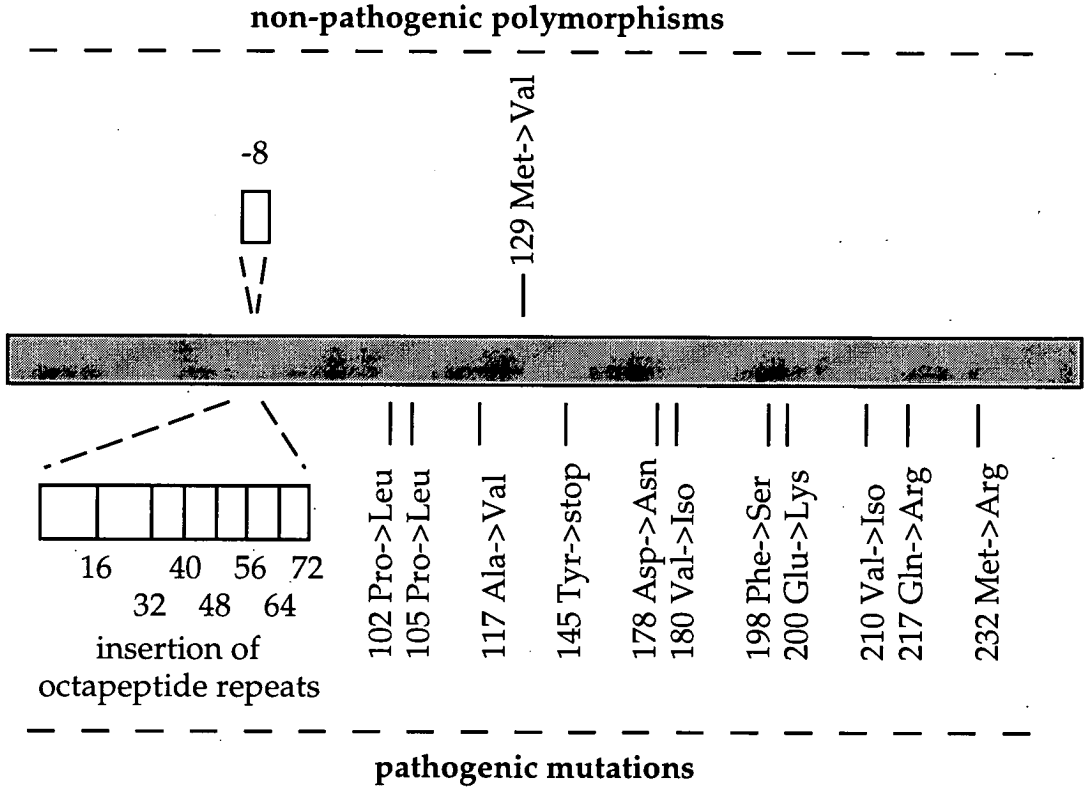


Figure 1.3 Pathogenic mutations and polymorphisms of PrP

great difficulty. Experimental studies on the pathogenesis of TSEs have been facilitated by the use of scrapie in rodents as a model. The incubation period and the time course of the disease can vary substantially depending on four main factors: the dose and the site of infection, the genotype of the mouse, and the strain of the infectious agent.

There is a different effective dose for each route of infection and the incubation period varies considerably with the dose of the agent (Kimberlin and Walker, 1979b; Kimberlin and Walker, 1978). Administration of the agent directly to the CNS - intraspinal (i.s.) and intracerebral (i.c.) injection - is more effective than the peripheral routes of infection - subcutaneous (s.c.), intraperitonial (i.p.) and intravenous (i.v.) (Kimberlin *et al.*, 1987b; Kimberlin and Walker, 1988a). The relative efficiencies of infection are i.v.>i.p.>s.c. The difference between i.v and s.c. is approximately 3,500-fold and between s.c. and i.c. 10,000-fold (Kimberlin and Walker, 1989a; Kimberlin and Walker, 1979b).

The peripheral routes of infection have been used to establish the dynamics of agent replication in different tissues before the invasion of the central nervous system. The involvement of the lymphoreticular system (LRS) was established in the 1960s, when it was demonstrated that following subcutaneous (s.c.) injection the first tissue to become infected is the spleen. The agent spreads to other tissues of the LRS, including the lymph nodes, thymus and submaxillary salivary glands (Eklund *et al.*, 1967). Subsequent studies confirmed this observation and demonstrated that in natural scrapie of sheep, the infection of the LRS precedes neural infection (Hadlow *et al.*, 1982).

The importance of the spleen was demonstrated in splenectomised, or genetically asplenic mice, by the prolongation of the incubation periods after intraperitonial (i.p.) injection (Fraser and Dickinson, 1970; Fraser and Dickinson, 1978; Kimberlin 1989a). However, the absence of the spleen did not affect all peripheral routes. When mice were infected by the oral route (intragastrically), neuroinvasion was proposed to occur through nerve endings in the gut or through Peyer's patches and the incubation period was shown to be unaffected by splenectomy (Kimberlin and Walker, 1989b). Upon s.c. infection of mink with TME the peripheral spread was confined to the lymph nodes and infectivity was detected in the rest of the LRS only after establishment of infection

in the CNS (Hadlow *et al.*, 1987). The efficiency of the s.c. route depends on the exact site of injection (Kimberlin and Walker, 1979b).

Intravenous (i.v.) injection is the most effective peripheral route, producing the shortest incubation periods, but there is no evidence supporting a direct infection of the CNS by a haematogenous route (Kimberlin and Walker, 1978). Capillary endothelial cells and liver show relatively high levels of infectivity immediately after i.v. infection, but they do not appear to sustain the infectious agent (Millson *et al.*, 1979; Diringer, 1984).

Although peripheral routes seem to have different efficiencies of infection with a specific scrapie strain (139A) the dose-response curves are similar, suggesting that pathogenesis follows common pathways (Kimberlin and Walker, 1978; Kimberlin and Walker, 1979b). Irradiation of mice had no effect on the pathogenesis, indicating that the cells of the LRS involved in the infectious process are probably long-lived and non-dividing (Fraser and Farquhar, 1987; Scott, 1993). Infectivity spreads from spleen and visceral lymph nodes along visceral autonomic nerves to the mid-thoracic cord (Kimberlin and Walker, 1988a). Direct spreading through nerve endings is also possible in the gut (Kimberlin and Walker, 1989b) and probably the skin, after scarification prior to contact with the infectious agent (Scott, 1993).

Once the agent has invaded the thoracic cord, there is a spread of the infection in the brain to specific, yet uncharacterised areas, termed clinical target areas (CTA) (Kimberlin and Walker, 1983). It has been shown that direct inoculation of the spinal cord produces shorter incubation periods than i.c. injection. This finding suggests that infectivity can target quicker the CTA by the spinal cord inervasions, than inoculation at a specific site in the brain (Kimberlin *et al.*, 1987b). This finding suggests that there are restrictions on the spread of the agent, depending on the different types of connecting neurons (Kimberlin and Walker, 1988a; Kimberlin and Walker, 1988b). Stereotaxic injection into various regions of the brain with different scrapie strains demonstrated differences in the incubation periods and lesion profiles, supporting the idea that infection in the CNS is restricted to specific neuroanatomical pathways (Kim *et al.*, 1987; Kim *et al.*, 1990b; Kim *et al.*, 1990a). Furthermore, ocular inoculation has demonstrated

that both pathology and infectivity can be targeted to neuroanatomical projections of the retina (Fraser and Dickinson, 1985).

The spread of infection depends on the site of entry of the infectious agent, which determines the pathway by which the CTA are reached, probably involving complex neuron-to-neuron steps (Kimberlin, 1990b). Splenectomy does not effect the i.c. route (Kimberlin and Walker, 1988a). The rate of the spread of infectivity in the CNS was estimated to be about 0.5-2 mm/day, which is much slower than that of conventional slow 'neuronal' viruses but similar to slow intra-axonal transport of cytoskeletal proteins (Kimberlin and Walker, 1988a; Kimberlin and Walker, 1988b). The suggestion that the number of scrapie permissive cells, or CTA, is limited is supported by the competition between strains after i.c. or i.p. coinjection (Dickinson *et al.*, 1972; Dickinson and Outram, 1979).

1.10 Strain variation and mutation of TSE agents and interaction with the host

Experimental transmission of scrapie to goats produced the first evidence that there were distinct strains of the infectious agent (Pattison and Millson, 1961; Pattison and Millson, 1962). Since then, over 20 strains have been isolated in experimental animals (mainly mice and hamsters) by serial passage of infectious agent from different sources, including a sheep, goats, cattle and mink (Bruce, 1993; Bessen and Marsh, 1992). The strains are distinguishable primarily on the basis of their incubation periods, in mice of a defined genotype. This discrimination is confirmed by the type, distribution and severity of neuropathological changes (Kascsak *et al.*, 1991; Bruce and Fraser, 1991). The pattern and degree of vacuolar degeneration of the neuropil is scored from coded sections in nine grey matter and three white matter brain areas to construct a 'lesion profile', characteristic for each strain (Fraser and Dickinson, 1973; Bruce and Fraser, 1991; Bruce, 1993). The strains also differ in other respects, such as the extent of cerebral amyloidosis produced, their neuroinvasiveness after peripheral infection, their clinical characteristics and their ease of transmission between species. Apart from the distinct phenotypic properties, the strains differ in

biochemical and molecular properties including resistance to thermal inactivation, the morphology of SAF, the sensitivity of PrP^{Sc} to proteinase K digestion and the molecular weight of PrP^{Sc} (Bruce and Dickinson, 1987; Kascsak *et al.*, 1991; Bruce and Fraser, 1991; Bessen and Marsh, 1992). For example, the two strains of TME, hyper (HY) and drowsy (DY), exhibit differences in certain properties of PrP^{Sc}, (PrP^{TME}), such as, sedimentation rate in the presence of N-laurylsarcosine, sensitivity to proteinase K digestion, solubility in detergent solutions, electrophoretic migration and immunoreactivity (Bessen and Marsh, 1992).

The differences in the pattern of degeneration in the brain between the strains (established in mice) suggests that they have different abilities to recognise and spread in particular sets of neurons (Bruce, 1993; DeArmond *et al.*, 1993; Hecker *et al.*, 1992). Apart from the characteristic 'lesion profile', the differences in pathology between strains can be demonstrated more clearly by the regional distribution of PrP^{Sc}. Immunostaining for PrP^{Sc} can reveal differences even within a certain brain region, for example, strains 87V, 79A, ME7 and 22A induce completely different distributions of PrP^{Sc} in mouse hippocampus (Bruce, 1993). The detection of PrP^{Sc} in brain sections, as well as the kinetics and patterns of its accumulation, were facilitated by the histoblot method (Taraboulos *et al.*, 1992a; DeArmond *et al.*, 1993; Hecker *et al.*, 1992). In these studies it was shown that Sc237 (or 263K), 139H and ME7H strains show distinct - although overlapping at some regions - patterns of PrP^{Sc} accumulation, suggesting that some neurons are capable of supporting replication of different strains, or, the same region might contain neurons with different susceptibilities to different strains (Hecker *et al.*, 1992; DeArmond *et al.*, 1993).

When a strain is passaged under standard conditions of dose and route of infection, it appears to have always a reproducible incubation period in each of three Sinc genotypes. For example, 22A strain has a shorter incubation period in $Sinc^{P7}$ than in $Sinc^{S7}$; in heterozygotes the incubation period is even longer. For the ME7 strain, the incubation period is shorter in $Sinc^{S7}$ than $Sinc^{P7}$ homozygotes, but in $Sinc^{S7}/Sinc^{P7}$ heterozygotes the incubation period falls between those observed for the homozygous genotypes (Bruce, 1993; Bruce and Fraser, 1991; Bruce *et al.*, 1991).

Some strains, such as 139A and 22C, retain the same properties when passaged through different host Sinc genotypes, while others, for example 22A, change. This change does not always happen at the first passage to the new genotype. With some strains, a gradual, reproducible selection of a new strain with a shorter incubation period has been observed after passage to a new Sinc genotype (Bruce and Dickinson, 1987; Bruce and Fraser, 1991). Other strains, for example 87A, are stable when passaged at a low dose in the same genotype, but mutate if passaged at a higher dose. The new strain, which exhibits shorter incubation periods, retains its characteristics, even when passaged at a high dose (Bruce and Dickinson, 1987; Bruce and Fraser, 1991).

The strains also appear to behave differently when transmitted to different species (Kimberlin *et al.*, 1989; Kimberlin *et al.*, 1987a; Bruce, 1993). Some remain completely unchanged, some are modified giving rise to new strains that remain stable in subsequent passages, while some change only after passage through certain species.

In summary, different strains are characterised by differences in pathogeneisity and host pathology. The Sinc genotype of the mouse host affects the incubation time of different strains in different ways, and in some cases brings about changes in the properties of the strain. Similar effects are observed on passage through different species.

1.11 The species barrier

When the infectious agent is passaged initially to a new species, a prolonged incubation period (usually accompanied by variations in the symptoms and histopathology) is observed, a phenomenon termed the species barrier (Pattison, 1965; Dickinson, 1976; Kimberlin and Walker, 1979a). However, the infectious agent can overcome the species barrier and on subsequent passages the incubation period decreases, until it stabilises for that species. Differences in the pathogenesis between the first and subsequent passages have commonly been observed. Usually the incubation period is longer after i.p. infection than after i.c. (see section 1.9), but in some interspecies passages the incubation periods for the two routes are similar (Bruce, 1993).

Passage of the infectious agent to a new species always results in an (initially) prolonged incubation period, even when the strain properties remain the same (Kimberlin *et al.*, 1987a; Kimberlin *et al.*, 1989; Bruce, 1993). This observation suggests that the host genome has a definite influence on the incoming agent. Experimentation involving transgenic mice has provided more evidence on this issue (see section 1.12.1).

1. 12 *In vivo* and *in vitro* models of TSEs

1.12.1 *In vivo* models of TSEs

Studies with transgenic mice have elucidated the role of the PrP gene in several aspects of the disease. Transgenic mice harbouring various numbers of copies of the hamster PrP cosmid clone (cosHaPrP) were shown to be susceptible to hamster-adapted scrapie (Sc237 strain, of the same origin as 263K), while non-transgenic mice were resistant (Scott *et al.*, 1989). Tg(cosHaPrP)81 mice containing 30-50 copies of the cosmid transgene showed an incubation period similar to that observed in hamsters (70-75 days). Tg(cosHaPrP)71 mice, containing 4-8 copies were also susceptible, but exhibited longer incubation periods (170 days). Neuropathology in these transgenic mice was characteristic of that observed in hamsters and hamster PrP^{Sc} was present in their brains. Both transgenic and non-transgenic mice were susceptible to mouse-adapted scrapie (Rocky Mountain Laboratory (RML) strain). However, problems were encountered with breeding transgenic lines generated with a minigene construct (based on the hamster PrP open reading frame) and the only established colony failed to express hamster PrP^{C} ($HaPrP^{C}$) (Scott *et al.*, 1989). The production of more Tg(cosHaPrP) lines established an inverse correlation between the incubation period and the amount of $HaPrP^{C}$ produced (Prusiner *et al.*, 1990). The titres in the brains of the transgenic mice were independent of the incubation periods. Inoculation of these mice with the RML strain produced mouse-adapted scrapie that could be passaged to mice but not to hamsters; neuropathology was characteristic of mice. Inoculation with the Sc237 strain produced hamster-adapted scrapie that could be transmitted to hamsters but not mice. Furthermore, when these Tg(cosHaPrP) mice

were inoculated with different strains of hamster-adapted scrapie (Sc237, 139H and ME7H), the properties of the particular strains were retained (Hecker *et al.*, 1992; DeArmond *et al.*, 1993) (see also section 1.10). These experiments demonstrate the importance of the PrP gene in controlling the incubation period, susceptibility and species barrier of the disease.

Transgenic mice carrying the mouse PrP transgene (Prn-p^b allele) modified to contain the GSS mutation (codon 102 Pro-> Leu in human, codon 101 in mice) have been generated (Hsiao *et al.*, 1991). High-expressing transgenic Tg(GSSPrP) lines, or Tg(MoPrP-P101L)H, showing approximately 8-fold overexpression of the transgene, developed spontaneous neurodegeneration and pathology indistinguishable from mouse-scrapie, but detection of PrP^{sc} was very rare (Hsiao *et al.*, 1991; Hsiao *et al.*, 1994). A low expressing line (2-fold higher than non-transgenic mice), designated Tg(GSSPrP)196, failed to develop spontaneous disease (Hsiao *et al.*, 1994). Subsequently, some Tg196 mice inoculated with brain homogenates from symptomatic Tg(MoPrP-P101L)H mice developed neurologic dysfunction between 226 and 716 days and the infectious agent from these mice was shown to be transmitted again to 83% of inoculated Tg196. Diseased Tg196 showed characteristic spongiform changes in the brain but no PrP^{sc} accumulation. The agent from Tg(MoPrP-P101L)H symptomatic mice was also transmissible to hamsters but not to Swiss CD-1 mice and PrP^{sc} was detected in some of the infected hamster brains. These experiments suggest that there are other factors apart from the PrP gene itself influencing the susceptibility and the course of the disease.

Two different strategies were used to disrupt the PrP gene by insertion of the neomycin resistance gene (Bueler *et al.*, 1992; Manson *et al.*, 1994). These mice, designated Prn-$p^{0/0}$ (or PrP null), had normal development and no obvious abnormal phenotype (Bueler *et al.*, 1992; Manson *et al.*, 1994). It was shown that they were resistant to infection by mouse-adapted scrapie, but became susceptible to hamster-adapted scrapie after introduction of hamster PrP transgenes (Bueler *et al.*, 1993; Prusiner *et al.*, 1993). These findings further demonstrated the importance of the PrP protein in disease susceptibility. Subsequent studies, based on electrophysiological techniques, revealed that PrP null mice have impairments in long term potentiation (LTP) and weakened $GABA_A$ (γ-aminobutyric acid type A) receptor-mediated fast synaptic

inhibition in the hippocampus (Collinge *et al.*, 1994). This phenotype was rescued by high, but not low, expression of a human PrP trangene (Whittington *et al.*, 1995). This deficit was not observed by another group (Lledo *et al.*, 1996), but has been independently reproduced (Manson *et al.*, 1995).

Studies with transgenic mice expressing chimeric PrP transgenes where different regions of the mouse PrP ORF were replaced with hamster equivalents (Scott *et al.*, 1992), demonstrated that a few amino acid changes in the primary sequence of the PrP protein produced by the transgene influence the susceptibility of the host to mouse- or hamster-adapted scrapie (Scott *et al.*, 1993). Tg(MH2M PrP) mice, in which mouse amino acids 94 to 188 were replaced with the hamster sequence (5 hamster specific amino acid substitutions) were susceptible to both RML and Sc237. Tg(MHM2 PrP) mice, with only two hamster specific substitutions between amino acids 94 and 131, were susceptible to RML only. Terminal infection of Tg(MH2M PrP) mice was associated with the deposition of chimeric MH2M PrP^{Sc} in the brain and subsequent transmission of infectivity was more efficient in transgenic Tg(MH2M PrP) mice, but also possible in both non-transgenic mice and hamsters. These studies support the idea that an homophilic interaction between PrP^{C} of the host and PrP^{Sc} of the inoculum is necessary for efficient infection.

Trangenic mice generated with a chimeric PrP gene, where mouse amino acids 96 to 167 were replaced by the human sequence (9 human specific amino acid substitutions), designated Tg(MHu2M PrP), were susceptible to inoculation with infectious brain homogenates from CJD patients, but only approximately 10% of transgenic mice expressing the human PrP, designated Tg(HuPrP) and non-transgenic mice developed neurologic dysfunction (Telling *et al.*, 1994). However, when the HuPrP transgene was combined with the homozygous mouse PrP null mutation, the mice became susceptible to human infectious agent, suggesting that the inhibition of transmission observed previously was due to the endogenous mouse PrP^{C} (Telling *et al.*, 1995). The susceptibility of Tg(MHu2M) was only slightly increased (Telling *et al.*, 1995). These findings led to the hypothesis that another molecule, a putative protein X, binds to a region of PrP^{C} (probably the C-terminal) and facilitates the interaction with PrP^{Sc}, while the central region of PrP^{C}

(including codons 96 to 167) interacts directly with PrP^{Sc}. The binding of this protein X to PrP^{C} would be promoted when the two proteins are from the same species. Therefore, in Tg(HuPrP) transgenic mice, the endogenous mouse PrP^{C} would interact with the mouse protein X inhibiting its binding to human PrP produced by the transgene and subsequently to PrP^{Sc} in the inoculum (Telling *et al.*, 1995).

However, two lines of the Tg(HuPrP) mice were reported to be susceptible to a single isolate of CJD and to two inherited TSEs (one with a 144-base pair insertional mutation in PRNP and FFI) by another research group (Collinge *et al.*, 1995), but the incubation periods were longer than those observed in mice expressing only human PrP (null background). These mice were shown to produce human PrP^{Sc}. Therefore, their ability to produce human PrP^{Sc} after challenge with the BSE infectious agent was assessed. In the Tg(HuPrP) mice (carrying also the endogenous MoPrP gene) only mouse PrP^{Sc} was detected, but confirmation that these mice are not producing any human PrP^{Sc}, by inoculations of brain homogenates into mice expressing only HuPrP, are still in progress. In mice expressing only the human PrP, no human PrP^{Sc} production was detected at 264 days after inoculation (Collinge *et al.*, 1995). However, it is too early to conclude that these mice are not susceptible to the BSE infectious agent.

Two PrP gene alleles, Prn-p^{a} and Prn-p^{b}, have been correlated with short and long incubation periods respectively, following infection with different strains, suggesting that the PrP sequence of the host influences susceptibility to mouse scrapie (see section 1.8). When heterozygous mice Prn-p^{a}/Prn-p^{b} were inoculated with the Chandler scrapie strain the incubation periods were longer than those observed in homozygous Prn-p^{a} mice (Carlson *et al.*, 1986; Carlson *et al.*, 1989). Homozygous Prn-p^{a} mice carrying several copies of a Prn-p^{b} transgene exhibited unexpectedly shorter incubation periods than non-transgenic controls (Westaway *et al.*, 1991). In an attempt to elucidate these observations, three different scrapie strains were tested in conjuction with a range of host genotypes expressing different ratios of Prn-p^{a} and Prn-p^{b} alleles (Carlson *et al.*, 1994). Congenic mice, PrP null mice (Bueler *et al.*, 1992) and transgenic mice carrying various copies of the Prn-p^{b} allele as well as F1 hybrids between the different mouse strains were used in these experiments. It was revealed that the level of PrP^{C} produced by the Prn-

p^a allele (MoPrP-A) was responsible for the incubation periods produced with the RML strain. Incubation periods were inversely related to the amount of MoPrP-A. Increase of MoPrP-B also resulted in shortening of the incubation periods, but to a lesser extent. In contrast, MoPrP-A expression had an inhibiting effect on transmission of the 22A and 87V isolates. Increasing MoPrP-A was related to increase of incubation periods, while the reverse correlation was observed with MoPrP-B. MoPrP-A expression conferred resistance to the 87V strain, regardless of MoPrP-B expression. These results suggest that allotypic interactions between PrP^C of the host and PrP^{Sc} of the inoculum influence the efficiency of transmission and the incubation period. Furthermore, the neuropathological features and PrP^{Sc} accumulation were characteristic for each isolate. However, differences observed in mice with the same Prn-p genotype inoculated by the 87V isolate suggested that a genetic locus distinct from Prn-p could be involved.

The transgenes used in all the transgenic experiments were based on cosmid clones (the mouse or the hamster), which were modified appropriately to contain cloning cassettes so as to permit the replacement of different ORF fragments. Because other DNA sequences apart from PrP gene were included in these transgenes, the involvement of other genetic factors in controlling several aspects of the disease could not be ruled out. This possibility was not further investigated since attempts to generate transgenic mice by using a hamster PrP ORF based construct (minigene) failed, suggesting possible absence of sequences necessary for appropriate PrP expression (Scott *et al.*, 1989).

In all the transgenic experiments, the mice contained multiple copies of the transgene and sometimes exhibited high levels of PrP^C expression. Spontaneous disease developed in some older animals carrying 120 and 60 copies of the Syrian hamster cosmid transgene, 36 and 31 copies of the Prn-p^b mouse cosmid transgene and high copy number of a sheep PrP cosmid transgene. Disease was not observed in animals with low transgene copy number (Westaway *et al.*, 1994a). The pathogenic changes included necrotising myopathy of the skeletal muscle, demyelination of the sciatic nerve and spongiform degeneration of the CNS. Low levels of proteinase K resistant PrP detected in a few samples, but were also detected in some young animals without any

symptoms of the disease, suggesting that the neuromyopathy was not due to TSE.

1.12.2 *In vitro* models of TSEs

Cell culture models of TSEs have provided information on the biosynthesis and metabolism of PrP^C and PrP^{Sc}, the conversion of PrP^C to PrP^{Sc}, agent replication, species specificity and strain variation.

A rat pheochromocytoma cell line (PC12) was shown to be able to sustain scrapie replication (strain 139A) when differentiated by addition of nerve growth factor (NGF) (Rubenstein *et al.*, 1984). NGF-treated PC12 cells develop a neuron-like morphology, stop dividing and show an induction of PrP mRNA expression (Wion *et al.*, 1988), but the difficulty of maintaining them has limited their use. Dose-dependent decrease of cholinergic enzyme activities (but not adrenergic) was demonstrated in 139A-infected PC12 cells (Rubenstein *et al.*, 1991). Strain variation was also shown in PC12 cells (Rubenstein *et al.*, 1992; Rubenstein *et al.*, 1994). Agent replication was much higher with the 139A strain than with ME7 (both mouse-adapted strains). No replication of the hamster-adapted 263K strain nor, paradoxically, of the rat-adapted 139R was observed.

Infection of a mouse neuroblastoma cell line (N2a) with mouse-adapted scrapie led to the establishment of persistently infected cells (Race *et al.*, 1987; Butler *et al.*, 1988). However, it was demonstrated that infectivity could be lost, especially under conditions of stress (Butler *et al.*, 1988). Another persistently infected cell line was established, designated HaB, derived from infected hamster brain (Taraboulos *et al.*, 1990b). Study of the biosynthesis and metabolism of PrP^C and PrP^{Sc} was based on these cell lines and uninfected N2a. It was shown that PrP^C acquires its glycophosphatidylinositol anchor in the endoplasmic reticulum, as well as high mannose glycans which are converted to more complex glycans in the Golgi apparatus (Caughey *et al.*, 1989). Mature PrP^C is attached to the membrane by a glycophosphatidylinositol moiety and can be released by treatment with proteases or PIPLC (Caughey *et al.*, 1990). The half life of PrP^C was shown to be approximately 3-6 hours (Caughey *et al.*, 1989). In contrast, PrP^{Sc} was shown to be proteinase K and PIPLC resistant and exhibited a very slow

turnover (half life over 15 hours) (Caughey *et al.*, 1989; Caughey *et al.*, 1990). It was demonstrated that the conversion of PrP^{C} to PrP^{Sc} is a post-translational change that probably occurs on the plasma membrane or at the endocytic pathway before exposure to lysosomal enzymes (Taraboulos *et al.*, 1992b; Borchelt *et al.*, 1992). An N-terminal truncation of PrP^{Sc} was observed after exposure to endosomal or lysosomal proteases (Caughey *et al.*, 1991). The site of accumulation of PrP^{Sc} appeared be in the cytoplasm (Taraboulos *et al.*, 1990b). In subsequent studies it was proposed that PrP^{Sc} accumulated in lysosomes (Taraboulos *et al.*, 1992b). No differences in the biosynthesis of the two isoforms has been detected (Caughey, 1993; Caughey *et al.*, 1989).

Several studies involving expression of recombinant PrP molecules in cell culture facilitated the study of the role of PrP mutations in PrP biosynthesis. Also, the importance of certain regions and mutations of PrP for the conversion and the species barrier was investigated. A series of different constructs was generated for this purpose; expression was carried out in insect cells and a range of mammalian cells (Scott *et al.*, 1988; Rogers *et al.*, 1991; Caughey *et al.*, 1988b; Scott *et al.*, 1992; Rogers *et al.*, 1993; Priola *et al.*, 1994; Chesebro *et al.*, 1993; Borchelt *et al.*, 1993; Priola *et al.*, 1995). Neuronal cell lines (especially mouse neuroblastoma cells) are used regularly for PrP expression and detection of PrP protein is done usually by immunoprecipitations. In the constructs used no PrP regulatory sequences were included (ORF-based constructs) and the expression of PrP was driven usually from strong viral promoters. The use of these constructs did not aid the exploration of the significance of untranslated and flanking regions of the PrP gene. Initial experiments showed that very high PrP mRNA expression was necessary to generate detectable levels of PrP^{C}. The use of recombinant vaccinia virus expression systems, retroviral vectors and stable transfections in conjunction with the use of immunoprecipitation to detect PrP made it possible to overcome this problem. Furthermore, it was noted that PrP processing can differ depending on the cell line (Scott *et al.*, 1988).

In order to investigate the regions that feature in the conversion of PrP^{C} to PrP^{Sc}, modified constructs based on the chimeric mouse PrP gene MHM2 (Rogers *et al.*, 1991; Scott *et al.*, 1992) were generated (Rogers *et al.*, 1993; Chesebro *et al.*, 1993; Taraboulos *et al.*, 1990a). MHM2 PrP protein contains a hamster specific epitope that can be recognised by the

monoclonal antibody 3F4 (Rogers *et al.*, 1991; Scott *et al.*, 1992). It can therefore be distinguished from endogenous mouse PrP^{C} and PrP^{Sc} produced in uninfected and infected N2a cells. It was shown that this chimeric PrP could undergo conversion to the proteinase K resistant form when expressed in scrapie infected N2a cells, while another chimeric PrP produced from the H3M construct, in which codons 50 to 188 of the mouse PrP are replaced by the hamster sequence, could not (Scott *et al.*, 1992). This result facilitated the identification of regions important for species specificity. Several modified MHM2 constructs were made and their ability to produce PrP^{C} that could be converted to PrP^{Sc} was tested in infected N2a cells. Deletion of amino acids 23 to 88, deletion of the octapeptide repeat region, addition of 1, 2, 4 and 6 octapeptide repeats and deletion of the GPI anchor did not affect the conversion of PrP to a proteinase K resistant state (Rogers *et al.*, 1993). In addition, removal of the glycosylation sites did not affect conversion (Taraboulos *et al.*, 1990a). Cell lines expressing mutant hamster PrP (GSS 102 mutation) did not cause conversion to PrP^{Sc} and expression of hamster PrP in N2a cells did not confer susceptibility to the hamster-adapted 263K stain (Chesebro *et al.*, 1993), in contrast to the results obtained from transgenic experimentation.

Initial attempts to convert PrP^{C} to the proteinase K resistant isoform in cell-free systems failed (Raeber *et al.*, 1992). Recently it was demonstrated that this conversion is possible after partial denaturation of PrP^{C} in guanidine-HCl and incubation with PrP^{Sc} (Kocisko *et al.*, 1994). The two strains of hamster-adapted TME that exhibit different proteinase K profiles (see 1.10), were shown to retain this strain specificity in a cell-free conversion reaction (Bessen *et al.*, 1995). Furthermore, the conversion was shown to be influenced by the species barrier between mouse and hamster (Kocisko *et al.*, 1995), consistent with the results from the *in vivo* studies.

Studies in cell culture have demonstrated that sulfated polyanions can inhibit PrP^{Sc} accumulation (Caughey, 1993; Caughey and Raymond, 1993). For example, Congo red (a disulfonated polyanion) has been shown to inhibit proteinase K-resistant PrP formation in scrapie-infected neuroblastoma cells (Caughey and Raymond, 1993; Caughey *et al.*, 1993; Caughey and Race, 1992). It has also been suggested that endogenous highly sulfated glycosaminoglycans (GAGs) are involved in the

conversion of PrP^{C} to PrP^{Sc} (Priola and Caughey, 1994; Caughey and Raymond, 1993; Caughey, 1993). Possibly GAGs guide and stabilise the interaction between PrP^{C} and PrP^{Sc}. Therefore, it has been hypothesised that sulfated polyanions bind to PrP^{C} and/or PrP^{Sc} and inhibit an interaction with cellular GAGs, which is crucial for the conversion of PrP^{C} to PrP^{Sc} (Caughey, 1993; Caughey and Raymond, 1993; Priola and Caughey, 1994). These findings suggest that sulfated glycans could potentially provide means of therapeutic intervention in human TSEs (Caughey, 1993; Caughey and Raymond, 1993).

1.13 Hypotheses on the nature and replication of the TSE infectious agent

It has been proposed that the infectious agent is devoid of nucleic acid and that it consists of a *pro*teinaceous *in*fectious particle, termed 'prion' (Prusiner, 1982) (see also section 1.4). The 'protein only', or 'prion hypothesis' is supported by a series of findings: PrP^{Sc} is the major component of the infectious agent and it is related to infectious titres, no nucleic acid has been identified and infectivity is resistant to procedures that modify, mutate, or destroy nucleic acids (section 1.4). Also, the importance of PrP in all aspects of the disease has been proved through genetic studies and the use of *in vivo* and *in vitro* models (sections 1.8, 1.12). It was therefore suggested that the infectious agent consists of an abnormal monomeric prion protein (PrP^{Sc}) which combines with a normal host protein (PrP^{C}) to generate an heterodimer. This would lead to the conversion of the normal protein to the abnormal form, followed by separation of the proteins and formation of monomeric infectious particles that could convert more normal protein (Prusiner, 1982; Prusiner, 1991). Another model suggests that disease depends on a nucleation mechanism where PrP^{Sc} is a 'seed' for a polymerisation reaction by which new PrP^{C} molecules are recruited and converted in more PrP^{Sc} molecules (Kocisko *et al.*, 1994). This reaction has been observed in amyloid fibril formation by other proteins (Jarrett and Lansbury, 1993), but it is known that TSEs are not always associated with the formation of PrP^{Sc} and amyloid plaques (sections 1.2, 1.12.1).

However, the existence of a wide variety of infectious agent strains that can retain their characteristics through passaging in different hosts and can 'mutate' when passaged in the same host (Bruce *et al.*, 1991; Bruce and Fraser, 1991) (see also section 1.10) brought about an alternative theory (the 'virino hypothesis'), which proposes that the infectious agent has its own genome and PrP^{C} could act as a protector or/and a receptor (Hope and Baybutt, 1991; Aiken and Marsh, 1990; Bruce and Dickinson, 1987; Bruce and Fraser, 1991). Reasons why the putative nucleic acid has not yet been identified could be that it is possibly extremely small in size, well protected, of very low abundance, or host encoded.

A model attempting to combine the controversial features of the different hypotheses has been proposed (Weissmann, 1991). This model suggests that the infectious agent (holoprion) consists of two components: PrP^{Sc} (apoprion), which could transmit the disease and initiate the pathogenic process itself and a genetic component (coprion), which could modulate the properties of PrP^{Sc} and determine the strain specific characteristics.

Strain diversity has also been proposed to reflect variations in the PrP protein due to different glycosylation patterns (Endo *et al.*, 1989; Prusiner and DeArmond, 1994; Weissmann *et al.*, 1993) or different structural conformations (Huang *et al.*, 1994; Stahl *et al.*, 1993; Prusiner, 1991; Prusiner, 1993b) (section 1.7.2), possibly combined with a selective permissiveness of various cell types to certain PrP isoforms (Hecker *et al.*, 1992; Prusiner and DeArmond, 1994; Weissmann *et al.*, 1993). These theories are currently being tested.

Recently, it has been proposed that another protein might be necessary for the interaction between PrP^{C} and PrP^{Sc} (Telling *et al.*, 1995). This protein X, encoded by the host, would interact with a region of PrP^{C}. A different part of PrP^{C} would interact with PrP^{Sc} and the formation of the complex would promote the conversion of PrP^{C} to PrP^{Sc} (section 1.12.1).

1.14 Aims and objectives

Previously, PrP transgenic animals were employed to demonstrate that a 40 kb cosmid clone, comprising of the entire hamster PrP gene and flanking sequences, could confer susceptibility to hamster TSE and model all aspects of the disease, including the incubation periods and lesion profiles produced by different strains (Scott *et al.*, 1989; Prusiner *et al.*, 1990; Hecker *et al.*, 1992) (section 1.12.1). Hamster PrP expression was driven by its own promoter and therefore directed to all tissues in which PrP is normally expressed. Investigation of the role of limited and specific PrP expression in controlling susceptibility, pathogenesis, and the species barrier, can be achieved only by directing expression of a PrP transgene to neurons in the brain and furthermore, to restricted areas of the brain. Also, in order to eliminate the contribution of any flanking sequences present in previously tested transgenes to the disease process, the use of the hamster PrP open reading frame (HaPrP ORF) as a minigene was considered for generation of transgenic mice. The main questions to be addressed were whether the PrP ORF could confer susceptibility, whether brain specific neuronal expression could confer susceptibility and whether region specific expression could confer region-specific susceptibility to the disease.

A previous attempt to express a PrP minigene had failed, but it was not clear whether that was due to the absence of adequate regulatory sequences, the influence of the integration site, or possible methylation of the transgene (Scott *et al.*, 1989) (see also section 1.12.1). Generation of multiple transgenic lines, together with the use of a characterised heterologous promoter and polyadenylation signal sequence, could help overcome that problem.

It was decided to use the neuron specific enolase (NSE) promoter for neuron specific expression and to further restrict expression to dopaminergic and adrenergic pathways by using the tyrosine hydroxylase (TH) promoter (section 2.2).

Apart from neurons, some PrP expression is detected in glial cells and it has been demonstrated PrP^{Sc} also accumulates in astrocytes and microglial cells, suggesting a possible role of these cells in the pathogenesis of the disease. Also, propagation of infectivity in the lymphoreticular system and particularly in the spleen seems to be

important for the establishment of the disease (section 1.9). Therefore, neuron-specific expression of hamster PrP could possibly be inadequate for replication of the hamster agent, or for PrP^{Sc} accumulation in the brain. It would be interesting to test whether glial cells are crucial for PrP^{Sc} formation and whether infectivity would be detected in the spleen after infection with the hamster agent. Also, the effectiveness of different routes of infection (direct injection in the central nervous system, or peripheral inoculation) could be determined. It is suspected that intracerebral, intrasciatic, or intraocular injection might overcome resistance to infection by peripheral routes.

In transgenic mice with hamster PrP expression in restricted regions of the brain some insight may be gained into the behaviour of the infectious agent and its interaction with PrP^{C} produced from the transgene. If the hamster agent was introduced stereotactically into the brain in a non-susceptible area (no hamster PrP expression), the ability of the agent to cross that area and target susceptible areas could be assessed. Introducing the agent into a susceptible area by stereotactic injection would test whether it would remain in that area or whether it would spread to unsusceptible areas. Also, if $HaPrP^{Sc}$ spread to areas of the brain in which $HaPrP^{C}$ was not expressed it would be interesting to see whether these areas would degenerate. If degeneration were to occur in regions that do not express hamster PrP but express endogenous mouse PrP, the rate of degeneration might differ in comparison to susceptible parts of the brain. This suggestion is supported by previous studies showing that the species barrier can be crossed (section 1.9, 1.10) and degeneration can occur, but only after very prolonged incubation periods (section 1.10). Another question that could be addressed is whether the lesion profiles of different hamster-adapted strains are affected by the restricted expression of hamster PrP^{C}.

If pathology appeared to be restricted to susceptible areas only, these areas would become effectively ablated. The role of the ablated brain regions could be investigated by phenotype analysis. This experiment could have more general application. The PrP gene could be linked to different promoters giving different expression patterns. Regions expressing PrP could be ablated by inoculation with the agent. One advantage of this approach is that the ablation could be at any chosen point in development.

CHAPTER 2

Generation and analysis of transgenic mice expressing the hamster PrP gene

2.1 Transgenic technology

Transgenic mice were first generated in the early 1980's; since then their use in biological research has increased exponentially (Aguzzi *et al.*, 1994). This technology has been used to create animal models of human diseases and assess the role of specific genes (or parts of genes) *in vivo* (Palmiter and Brinster, 1986; Aguzzi *et al.*, 1994). Transgenic mice are generated by microinjection of a DNA construct into the pronuclei of fertilised eggs, which are then reimplanted into a foster mother and allowed to develop to term (Palmiter and Brinster, 1986). The DNA generally integrates at a single, random chromosomal site and, when multiple copies integrate, they usually form a tandem head-to tail array. In most cases this foreign DNA (transgene) is stably transmitted to the offspring. Transgenic technology is now applicable to a wide range of species including rat, rabbit, sheep, goat, cattle, fish and insects (Lathe and Morris, 1994).

The gene of interest can be introduced as a transgene either under the control of its own regulatory elements or those of a foreign gene. To assess the elements required for high-level and correct expression, different putative control regions of a gene are used to drive the expression of a reporter gene. Tissue specific transgenes can be expressed correctly if they carry all necessary elements, but expression can be influenced by the site of integration: abnormal expression patterns are often detected depending on the sensitivity of the promoter to the chromosomal context (Palmiter and Brinster, 1986). Integration near specific enhancer sequences might cause ectopic expression, while some transgenic constructs do not express at all, probably due to integration into heterochromatic regions. Some constructs fail to express because of interference from vector sequences, other constructs may contain inappropriate (and often unknown) binding sites for negative regulatory factors, or may lack sequences such as topoisomerase binding sites,

nuclear matrix-attachment sites, or origins of replication (Palmiter and Brinster, 1986). Expression levels sometimes correlate with the copy number of the transgene but other times not, indicating that many transgene constructs lack distal elements (e.g. locus control regions (LCRs)), necessary for copy-number dependent gene expression.

Constructs that are functional in tissue culture do not always express reliably in transgenic mice. This might be due to developmental regulation of the transgene, or the influence of the integration site (Palmiter and Brinster, 1986). It has also been shown that a larger fragment of DNA than that used in tissue culture may be necessary for *in vivo* expression (Palmiter and Brinster, 1986).

Generally, large transgene constructs are recommended for position-independent gene expression. Such constructs are likely to include more regulatory elements of the selected gene. For this reason cosmid clones (inserts up to ~40 kb), recombinant P1 bacteriophage (up to 100 kb), yeast artificial chromosomes (YACs) (usually ~300-500 kb, >1 Mb possible) and bacterial artificial chromosomes (BACs) (up to 350 kb) can be used (Lathe and Mullins, 1993). Alternatively a defined promoter can be used in conjuction with the isolated coding region of the gene of interest. The expression of these constructs is however more likely to be affected by the site of integration and it is possible that elements usually necessary for the expression of the gene will be accidentally excluded. It has been also shown that the presence of introns can enhance expression of certain genes (Brinster *et al.*, 1988). Other approaches applied in order to achieve high level, specific and site-independent expression include co-integration strategies and the use of LCRs, or matrix-attachment regions (MARs) (Lathe and Mullins, 1993).

Transgenic mice carrying foreign PrP genes have provided insights into the role of PrP in susceptibility and incubation period, interspecies transmission and pathogenesis of TSEs (section 1.12.1). The study presented in this chapter was an attempt to expand and further test previous findings by using transgenic technology (section 1.14).

2.2 The choice of promoter

The 5' flanking sequences of the rat neuron specific enolase (NSE) gene and the rat tyrosine hydroxylase (TH) gene were chosen to drive hamster PrP expression to specific areas of the brain in transgenic mice. The 5' regulatory elements of each of these genes had been characterised in a transgenic context and were readily available.

2.2.1 Neuron specific enolase (NSE) promoter

Neuron-specific enolase (NSE) is one of the three types of isozymes of enolase (2-phospho-D-glycerate hydrolase), an enzyme involved in glycolysis. Each isozyme is composed as a homodimer. The $\alpha\alpha$ homodimer is the non-neuronal enolase (NNE) expressed in glial cells, the ßß isozyme is localised in mature muscle and the $\gamma\gamma$ isozyme is expressed in terminally differentiated neurons and neuroendocrine cells (Sakimura *et al.*, 1987).

Studies on rat and monkey have demonstrated that NSE and NNE are developmentally regulated (Schmechel *et al.*, 1980; Marangos *et al.*, 1980). In embryonic and early postnatal brain NNE levels are high and NSE levels very low. NSE expression appears after neurogenesis starts, increases as neurons mature functionally and morphologically and slowly reaches adult levels, resulting in NSE being one of the most abundant proteins of the brain. NNE levels decrease when NSE expression begins and then increase again to adult levels.

The rat NSE cDNA has been isolated and the developmental expression has been characterised (ForssPetter *et al.*, 1986). The rat NSE gene has been isolated and its structure has been analysed (Sakimura *et al.*, 1987). *In vitro* expression studies indicated that about 1.5 kb of 5' flanking sequence contains elements able to drive efficient transcription of NSE (Sakimura *et al.*, 1987). A sequence that resembles the TATA box (TCTATAGG) has been identified at positions -142 to -135 and an enhancer core-like sequence (GGGTTATTA) has been identified at position -350 in respect to the transcription initiation sites, upstream exon one. A typical CAAT box was absent. The region immediately

upstream of the cap site contains many GC-box like sequences thought to be important in regulation of transcription.

A transgene containing 1.8 kb of the rat NSE 5' flanking sequence fused to the ß-gal reporter gene was created to test whether these sequences could direct tissue-specific expression (ForssPetter *et al.*, 1990). The analysis of the transgenic mice generated demonstrated that the cis-acting regulatory sequences of the NSE promoter could confer neuron specific expression although some ectopic expression was detected in the testis. It was shown that ß-gal mRNA expression was developmentally regulated in a similar way to that of the endogenous NSE mRNA, but in some lines the levels of transgene RNA were much lower than that of the endogenous gene.

Recent experiments using different fragments of the rat NSE 5' flanking sequence fused to the CAT gene demonstrated that the cis-acting regulatory elements responsible for enhanced CAT expression in neuronal cell lines and primary cultures were localised in the region of -190 bp to -1500 bp upstream the transcription initiation site and also in a 500 bp portion of intron 1 (Sakimura *et al.*, 1995). Enhanced expression was detected only when both intron 1 and 5' flanking sequences were used.

For the generation of transgenic lines that would express hamster PrP specifically in neurons in the brain, the 1.8 kb rat NSE fragment was used, since it had been demonstrated that it was adequate to drive neuron-specific expression in the brain in a transgenic model. If ectopic expression was observed in any of the lines created, its effect on the course of the infection could be further investigated by experimenting with different routes of infection.

2.2.2 Tyrosine hydroxylase (TH) promoter

TH catalyses the oxidation of tyrosine to L-dihydroxyphenylalanine (L-DOPA) (Kandel *et al.*, 1991). This is the first and rate limiting step in the synthesis of catecholamines: dopamine, noradrenaline, and adrenaline. The expression of TH is limited to discrete populations of cells in the central and peripheral nervous systems. In the central nervous system (CNS), TH-expressing neurons include adrenergic and

noradrenergic cells of the brainstem, dopaminergic cells of the midbrain (containing the ventral tegmental area and the substantia nigra), the diencephalon (periventricular and hypothalamic nuclei), retinal amacrine cells and dopaminergic cells in the olfactory bulb (Banerjee *et al.*, 1992). TH-expressing neurons in the brain are involved in such functions as motor, neuroendocrine, biorhythm, feeding, mating, emotion, learning and memory, and sleep-wake cycle regulation (Kaneda *et al.*, 1991; Grima *et al.*, 1987). In the peripheral nervous system (PNS), TH expression is mainly limited to sympathetic ganglia and the adrenal medullary chromaffin cells. It is involved in the sympathetic function of PNS (Banerjee *et al.*, 1992; Kaneda *et al.*, 1991).

Long term alteration of TH activity is due to transcriptional stimulation of the TH gene. Glucocorticoid, cAMP, nerve growth factor and epidermal growth factor have been reported to increase TH activity through transcriptional regulation of the TH gene (Lewis *et al.*, 1987; Gizang-Ginsberg and Ziff, 1990; Lewis and Chikaraishi, 1987).

Highly conserved regions, likely to play an important role in the regulation of TH gene expression, were identified by comparison of the 5' flanking regions of human and rat TH genes, (Le Bourdelles *et al.*, 1988).

Expression studies involving deletions and site directed mutagenesis on the 5' flanking sequence of the rat TH gene have revealed that sequences from -205 to -182 comprise an AP1 motif and a partially overlapping 20 bp dyad symmetry element which contains an E box core (CANNTG). These elements are required both *in vivo* and *in vitro* for efficient and cell specific expression of TH (Sung Ok and Chikaraishi, 1992).

Transgenic mice generated with 4.8 kb of the 5' flanking sequences of the rat TH gene linked to the CAT reporter gene predominantly exhibit correct tissue-specific expression in the CNS and to a lesser extent in the periphery. This study was based on one transgenic line of 4 lines analysed as only one expressed the transgene. Transgene expression was measured by CAT enzyme activity assay of dissected brain regions. CAT activity was detected in all the expected areas containing TH-positive cells, although some ectopic but low level expression was detected in other areas such as the striatum, anterior olfactory nucleus and cortex. The highest expression was observed in the olfactory bulb, which

contains the majority of the CNS TH-expressing cells. Also, developmental expression of CAT was found to parallel the postnatal rise in endogenous TH in the olfactory bulb (Banerjee *et al.*, 1992).

Several transgenic mouse lines generated with a 5.3 kb fragment of the rat TH promoter driving the thymidine kinase (TK) gene exhibited different expression patterns of TK in the brain, demonstrated by *in situ* hybridization and immunocytochemistry, probably depending on the site of integration of the transgene. Some lines showed the expected expression in the nigrostriatal pathway, including the ventral tegmental area, substantia nigra and locus ceruleus, but additional expression in non-catecholaminergic regions (frontal cortex, colliculi, cerebellum) was detected. Other lines showed only low level ectopic expression (cerebellum, anterior cortex) and some did not express at all (Dr R.Al-Shawi, personal communication).

In recent studies 0.15 kb, 2.4 kb, and 9.0 kb fragments of 5' flanking sequence of the rat TH gene were fused to the E. coli lacZ (ß-galactosidase) reporter gene in order to identify TH promoter elements that could confer *in vivo* tissue specific expression. Transgenic mice bearing 9.0 kb, but not the smaller constructs with either 2.4 kb or 0.15 kb of 5' flanking sequence, displayed specific expression of ß-galactosidase at levels equivalent to the endogenous TH, in the central catecholaminergic cells and, to a lesser extent, in the adrenal gland (Min *et al.*, 1994).

To achieve region-specific expression in the brain of transgenic mice, the 5.3 kb fragment of the TH promoter was chosen, since it was the most thoroughly characterised at the time and immediately available. The fact that the expression patterns in the brain produced by using this fragment were different between transgenic lines was considered to be an advantage, since the main aim was to achieve region specific expression without having preferences about the exact regions. Transgenic lines expressing the hamster PrP in discrete, but different areas of the brain provide more options for testing the participation of certain cell types in the course of disease. If any of the transgenic lines generated expressed PrP only in the catecholaminergic pathways of the brain, it would be the only region that would degenerate after infection with the agent.

2.3 DNA constructs for the generation of transgenic mice

The following sections describe the cloning strategies used to link the promoter regulatory elements of either the NSE or TH genes to the HaPrP ORF and the Simian virus 40 (SV40) polyadenylation (poly(A)) sequences.

Two constructs, pNSE HaPrP and pTH HaPrP, were generated by several cloning steps and were used for the production of transgenic mice (Tg).

The plasmid vector pBluescriptIIKS- (Stratagene, appendix 2) was used as a basis for the constructs. pBluescriptIIKS- was chosen because the entire transgene construct can be excised from the multiple cloning site (MCS) by a single digest using a rare restriction endonuclease, BssHII. Also, pBluescriptII KS- contains the lacZ gene downstream of the MCS, which is useful for selection of recombinants.

2.3.1 Cloning of the HaPrP cDNA: generation of pBluescript HaPrP

The HaPrP was chosen for generation of Tg mice because information gained from previous transgenic studies could be exploited. Infection by different strains of infectious agent has been studied and the incubation periods and lesion profiles have been characterised. Also, the fact that hamster-specific anti-PrP antibody is available, facilitates detection of the transgene-encoded PrP protein.

A 1.9 kb HaPrP cDNA was obtained from pHG327[Sst]/f1-QB expression vector (Dr N. Robakis, unpublished vector, appendix 2). The HaPrP cDNA clone was excised by BamHI restriction endonuclease digestion and subcloned into the BamHI site of pBluescriptIIKS- for further characterisation. Attempts to sequence that fragment with the T3 and T7 sequencing primers (appendix 1) flanking the pBluescriptIIKS- polylinker failed because of nucleotide repeats around the cloning sites of the HaPrP cDNA. That was probably due to the terminal transferase tailing procedure used for the initial cDNA cloning (Old and Primrose, 1985). Subsequently, internal sequencing primers, 569G, 241V, 325S, 324S, 164N (appendix 1), corresponding to nucleotides 303-288, 526-490, 263-281, 556-573, 755-725 respectively (numbers according to the start of the

HaPrP cDNA; number 1=10th nucleotide upstream of the initiating ATG) were used to overcome that problem and the complete sequence of the cDNA was determined.

In order to avoid the repeats at either end of the cDNA and most of the 3' UTR sequence, PCR primers were designed including convenient restriction sites for subcloning. The expected size of the amplified fragment is 900 bp and it contains the HaPrP ORF and 168 bp of the 3' UTR. The forward primer, 485W (appendix 1), included a HindIII restriction site and was designed to match the 15 first nucleotides (10 nt upstream of the initiating ATG and 5 nt of HaPrP ORF) of the 5' end of the cDNA starting just beyond the polyG repeat sequence present in the HaPrP cDNA clone. The reverse primer, 484W (appendix 1) included sequences matching the 3' UTR at an internal position, 153-168 nt distal to the TGA stop codon and introduced an EcoRI site. The PCR product was gel purified (section 7.6.2) and cloned between the HindIII and EcoRI sites of pBluescriptIIKS-, generating the construct pBluescript HaPrP (Figure 2.3.1). The insert in pBluescript HaPrP was sequenced and confirmed to be free of any mutations which may have been introduced during the PCR procedure.

2.3.2 Generation of pNSE HaPrP construct

The rat NSE promoter (1.8 kb fragment) was derived from the pNSE lacZ construct (ForssPetter *et al.*, 1990, appendix 2). That was used to generate pNSEPrP.3 construct (Dr O. Windl and Dr P. Estibeiro, see appendix 2). The HuPrP ORF fragment of pNSEPrP.3 was replaced by the HaPrP fragment (derived from pBluescript HaPrP, see Figure 2.3.1) to generate pNSE HaPrP (Figure 2.3.1, appendix 2).

2.3.3 Generation of modified pBluescriptIIKS- vector (pBluescript ΔPS.N.A.)

The polylinker of pBluescriptIIKS- was modified by deletion of a part of the MCS (PstI-SacI) and insertion of a HindIII-NotI-EcoRI (HNE) adaptor, to generate pBluescipt ΔPS.N., as shown in Figure 2.3.2. The two

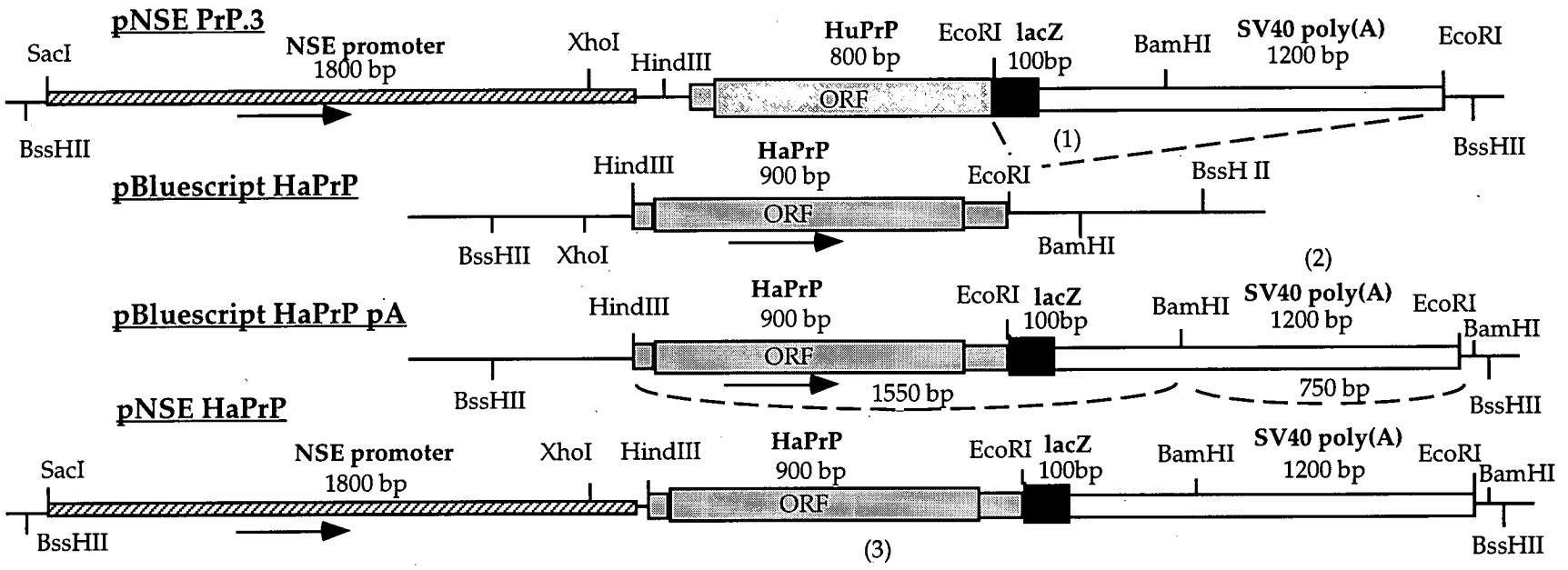


Figure 2.3.1 Generation of pNSE HaPrP construct

(1) pNSEPrP.3 and pBluescript HaPrP constructs were cleaved by EcoRI restriction endonuclease digest.

(2) The EcoRI-EcoRI lacZ-SV40 poly(A) fragment was subcloned into pBluescript HaPrP. Correct orientation was tested by Bam HI digest, which should give a 750 bp SV40 fragment and a 4350 bp vector fragment.

(3) The HindIII-BamHI fragment of pBluescript HaPrP pA construct, carrying the 900 bp HaPrP fragment and 550 bp fragment including SV40 and lacZ sequences was cloned into pNSEPrP.3, which was previously digested with HindIII and BamHI. This cloning step resulted in the substitution of the HindIII-BamHI fragment of pNSEPrP.3 containing the human PrP fragment with the equivalent fragment from pBluescript HaPrP pA, to create the construct pNSE HaPrP, used for the generation of transgenic mice.

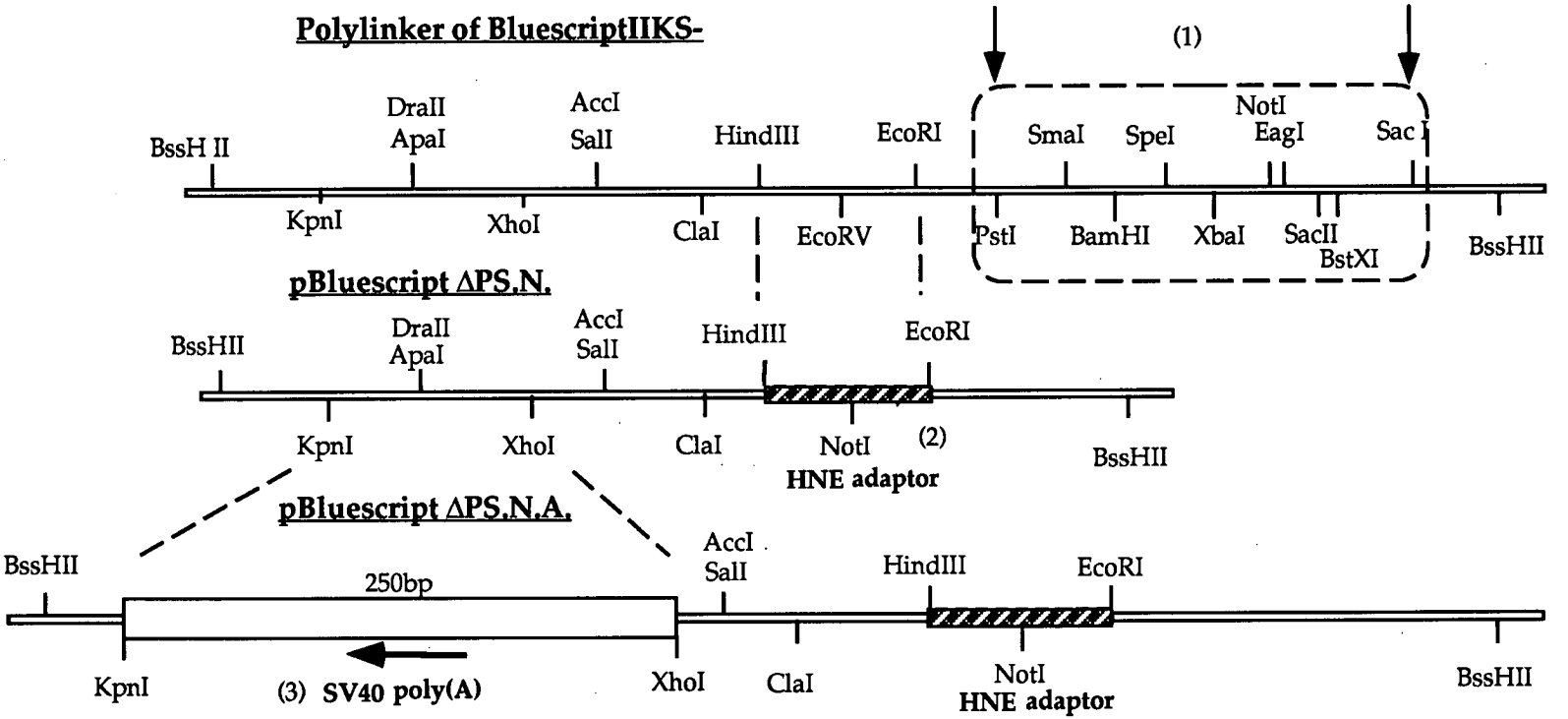


figure 2.3.2 Generation of 'cloning' vector pBluescriptΔPS.N.A.

(1) The Pst I-Sac I fragment of the polylinker was deleted (ΔPS vector) so that the sites in that region would not interfere with subsequent cloning steps.

(2) A HindIII-EcoRI adaptor including a NotI site (pre-annealed HNE I and HNE II oligos), was inserted between the existing HindIII and EcoRI sites (ΔPS.N. vector).

(3) The SV40 polyadenylation signal was inserted between the KpnI and the XhoI site (ΔPS.N.A. vector).

oligos used to create the HNE adaptor were HNEI and HNEII (appendix 1).

PCR amplification of a 250 bp fragment containing only SV40 poly(A) sequences from pUC19-SV40 vector, using primers containing appropriate restriction sites was then used. pUC19-SV40 vector is based on pUC19 (New England Biolabs) and contains SV40 sequences including the poly(A) addition sites (between the BamHI and BclI sites of SV40). The forward primer, 076X (appendix 1), contained an XhoI site and included SV40 sequences immediately downstream of the BclI site. The reverse primer, 487W (appendix 1), contained a KpnI site and included SV40 sequences just upstream of the BamHI site. The 250 bp SV40 fragment was cloned as a XhoI-KpnI fragment into pBluescipt ΔPS.N. to generate the construct pBluescript ΔPS.N.A. (Figure 2.3.2, appendix 2). After cloning, the fragment was sequenced in order to confirm that no mutations had been introduced by PCR amplification.

2.3.4 Cloning the rat TH promoter in pBluescript ΔPS.N.A.

The cloning steps necessary for subcloning the rat TH promoter from the pTH 5 kb CAT construct (gift from Dr J.Mallet, unpublished construct, appendix 2) into the ΔPS.N.A. vector are presented in Figures 2.3.3 and 2.3.4. pUC19 (New England Biolabs, appendix 2) was used for intermediate cloning steps. An EcoRI oligonucleotide linker (EcoRI l. oligo, appendix 1) was used for cloning steps (1) and (5). By using this linker sequence, the SalI site at the 5' end of the promoter is retained, but the SmaI site of the polylinker of pUC19 is destroyed. The completed construct, pBluescript ΔPS.N.A.TH, is shown in Figure 2.3.4 (6).

2.3.5 Generation of the pTH HaPrP construct

The HindIII-EcoRI HaPrP fragment was excised from pBluescript HaPrP (Figure 2.3.1) and subcloned into pBluescript ΔPS.N.A.TH (Figure 2.3.4) after the addition of HindIII linkers (HindIII l. oligo, appendix 1), as demonstrated in Figure 2.3.5.

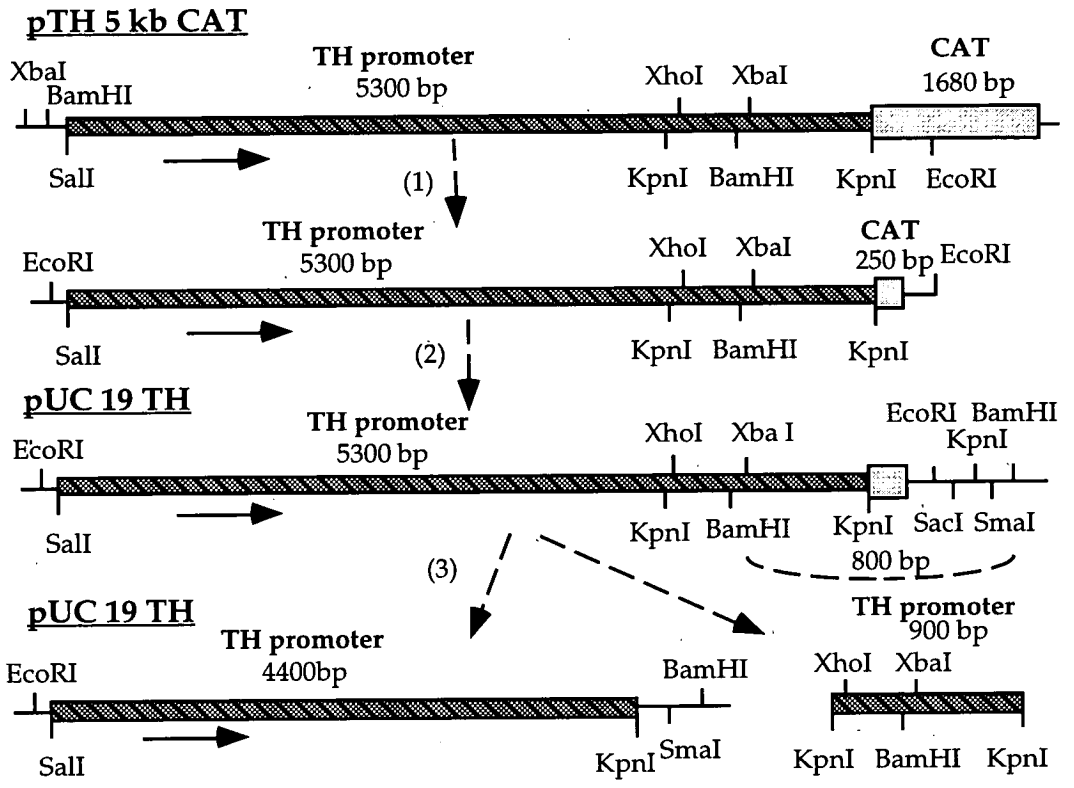


Figure 2.3.3 Subcloning of TH 5.3 kb fragment

(1) TheTH promoter was excised from the pTH 5 kb CAT construct by a SalI and EcoRI restriction digests and EcoRI linkers were added.

(2) The TH promoter and a small fragment remaining from the CAT gene were subcloned into the EcoRI site of pUC19. The orientation of the fragment was tested by BamHI restriction digest (unique site in the TH promoter and in the polylinker of pUC19). Correct orientation should give an 800 bp fragment, containing the CAT fragment and a fragment of the TH promoter and a 7500 bp fragment containing the large TH fragment and vector sequences.

(3) The remaining KpnI-EcoRI fragment of the CAT gene was removed by KpnI digest. The pUC19 TH vector, still carrying 4400 bp of the promoter sequence was recircularised. The 900 bp KpnI-KpnI fragment of the TH promoter, which was removed by the KpnI digest, was subsequently used for subcloning into pUC19 TH.

(continuing in Figure 2.3.4)

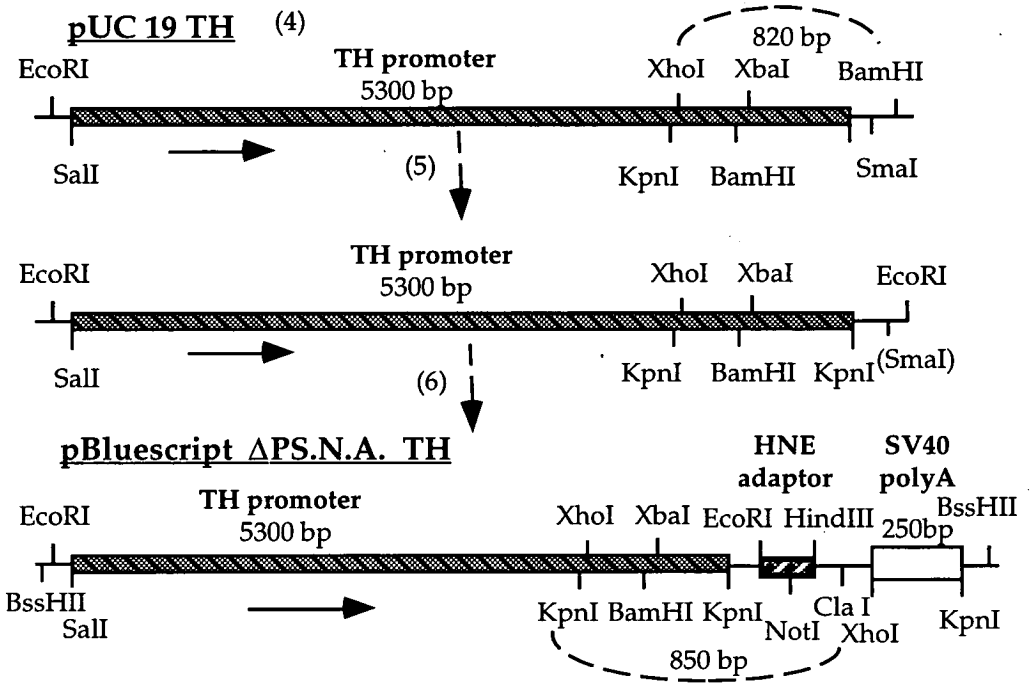


Figure 2.3.4 Subcloning of TH 5.3 kbfragment

(continuing from Figure 2.3.3)

(4) The 900 bp Kpn I- Kpn I TH promoter fragment was subcloned into pUC 19 TH. The orientation of the fragment was determined by double digest,with Xho I (unique site in the 900 bp fragment of TH) and Sma I (in the polylinker of pUC19). Correct orientation gave a 820 bp fragment containing most of the small TH fragment and a 7200bp fragment containing the rest of the TH promoter and vector sequences.

(5) The TH promoter was cleaved with Eco RI and Sma I and Eco RI linkers were added.

(6) The TH promoter was cloned into the Eco RI site of pBluescript II KS- ΔPS.N.A. and the orientation was determined by restriction digest with Xho I (unique site in TH promoter and in the polylinker of ΔPS.N.A.) which should give an 850 bp TH fragment and a 6700 bp fragment, containing the rest of the TH promoter and vector sequences .

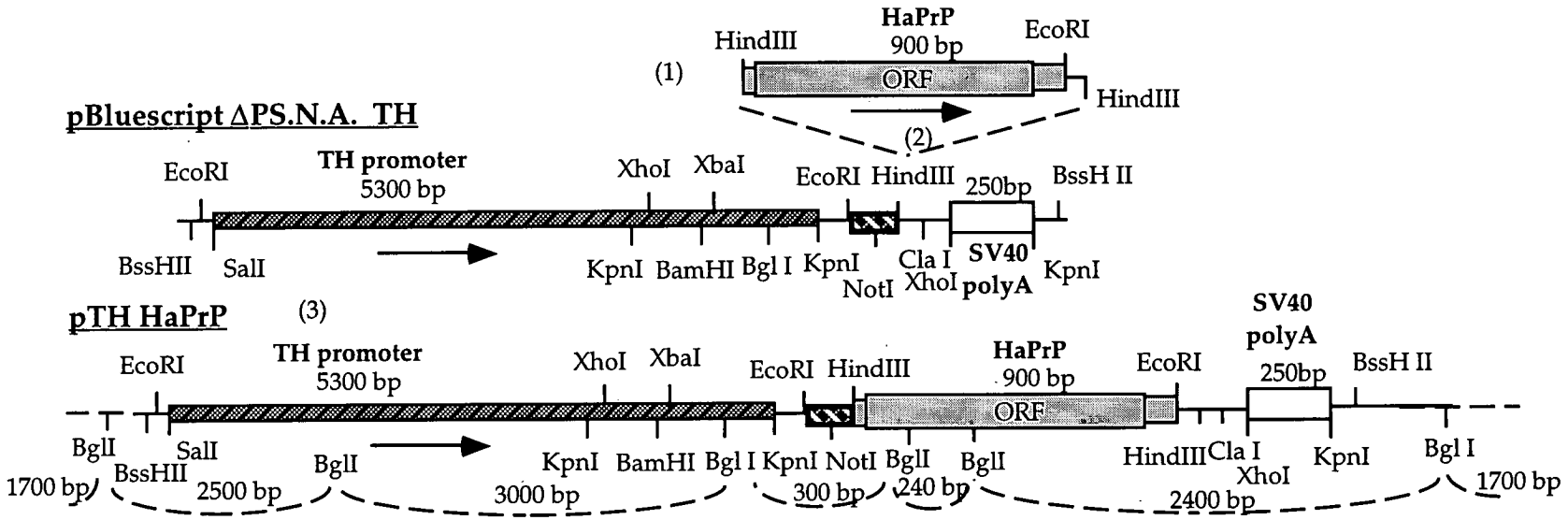


Figure 2.3.5 Generation of pTH HaPrP construct

(1) The HaPrP HindIII-EcoRI fragment was excised from pBluescript HaPrP construct and HindIII linkers were added.

(2) The HaPrP fragment was cloned into the HindIII site of pBluescript ΔPS.N.A., generating the construct pTH HaPrP (3). The orientation was tested by a BglI restriction digest, which cleaves pBuescriptII KS - twice, TH promoter twice and HaPrP twice. The correct orientation should give: a 2400 bp fragment containing HaPrP, SV40 and vector sequences, a 240 bp fragment of HaPrP, a 300 bp fragment containing HaPrP, TH and polylinker sequences, a 3000 bp TH promoter fragment, a 2500 bp fragment including TH promoter and vector sequences and a 1700 bp fragment containing the rest of the vector.

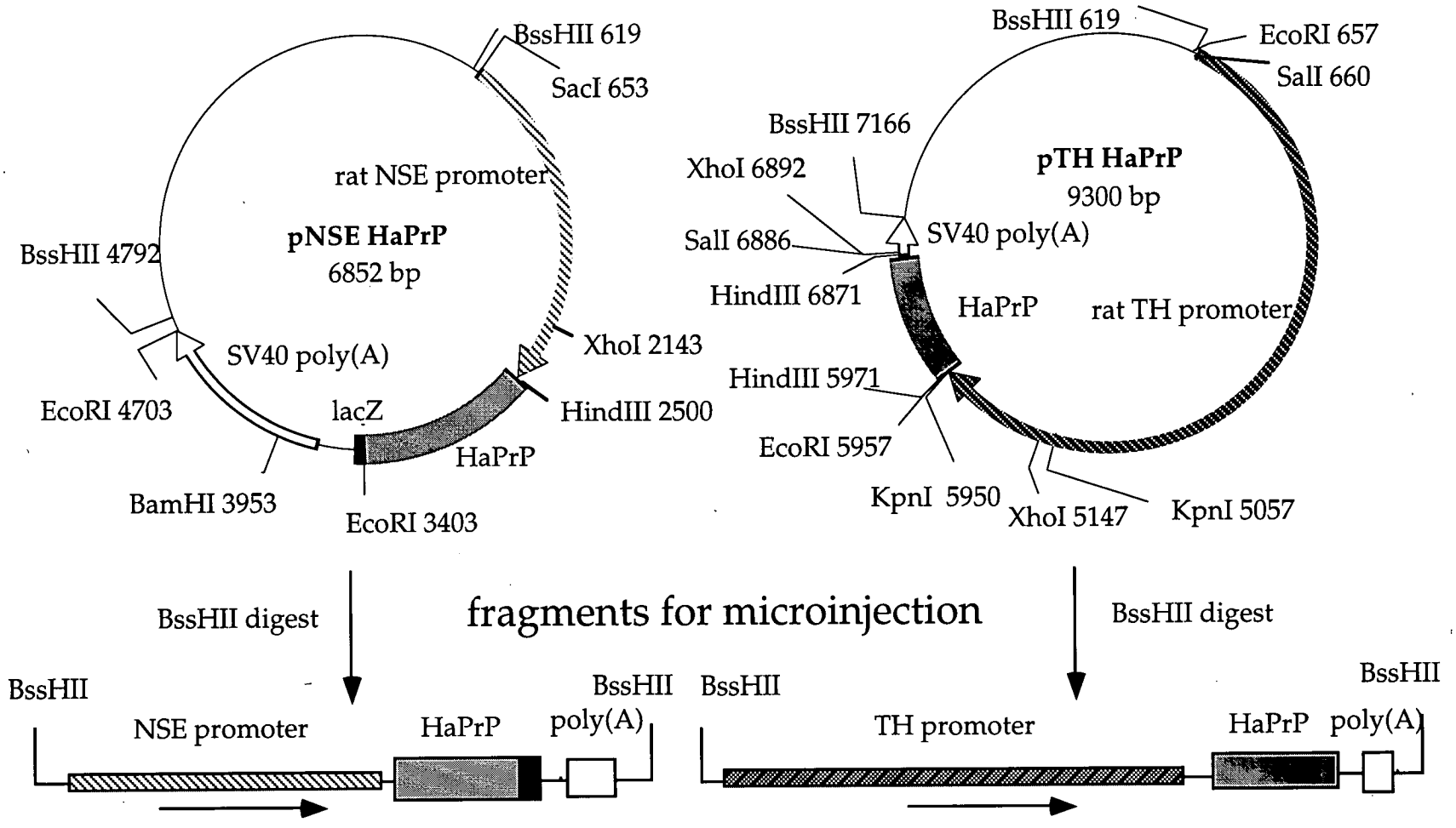


Figure 2.3.6 pNSE HaPrP and pTH HaPrP constructs.

NSE HaPrP and TH HaPrP fragments used for the generation of transgenic mice, are produced by a single BssHII digest from pNSE HaPrP and pTH HaPrP constructs.

By addition of the HindIII linker the EcoRI site on the 3' of the HaPrP fragment was retained.

After completion of all cloning steps, both pNSE HaPrP and pTH HaPrP constructs (appendix 2) were sequenced to confirm integrity and orientation of constituent fragments.

With a BssHII restriction endonuclease digest each of these constructs gave the fragment used for microinjection into fertilised mouse eggs (schematically presented in Figure 2.3.6), for the generation of transgenic animals.

2.4 Generation of transgenic mice

Purified TH HaPrP and NSE HaPrP BssHII fragments (Figure 2.3.6) were microinjected into fertilised mouse eggs (by Dr P. Estibeiro) to generate transgenic (Tg) mice as described in section 7.31.2. Mice carrying the transgene were identified either by PCR (section 7.6.11), or by dot blot (section 7.15) of DNA recovered following tail, or ear, biopsy (section 7.7.1). The dot blot method gave more reproducible results, it was easier to perform for large numbers of samples and the blots could be used for hybridisation with different probes.

In using PCR to identify Tg founder mice, the forward primer, HaP1 (appendix 1), was located in the HaPrP ORF, 540 bp downstream of the initiating ATG. The reverse primer, tgSV40.1 (appendix 1), was located upstream of the BamHI site in the SV40 sequence. These primers could be used for identifying Tg mice generated with both constructs. The expected size of the PCR fragment is 910 bp in the NSE HaPrP transgenic mice and 630 bp in the TH HaPrP transgenic mice, as presented in Figure 2.4.1.

Founder mice were crossed to non-transgenic F1 mice and the transgene was maintained in an heterozygous state. Four Tg lines were generated with the NSE HaPrP transgene (Tg NSE 14, 17, 20, 23) and 3 Tg lines with the TH HaPrP transgene (Tg TH1, 10, 15) (Table 2.4.1). Tg NSE 23 founder did not give rise to any transgenic progeny, indicating that probably it was chimeric.

Figure 2.4.1 Identification of Tg founder mice by PCR amplification.
PCR amplification (section 7.6.11) from mouse genomic DNA (section 7.7.1) and schematic presentation of the expected PCR products. The PCR products were electrophorised on a 1% agarose gel (section 7.5).
A. The size of the PCR product is 910 bp in the Tg NSE mice. Identification of the founder Tg NSE 14 (lane 7) is shown here.
B. The size of the PCR product is 630 bp in the Tg TH mice.
Identification of the founder Tg TH 1 (lane 2) and Tg TH 10 (lane 11) is shown here.
Lower intensity PCR products of other than expected sizes were considered as artefacts. Lane 1 in both gels is the 1 kb ladder.

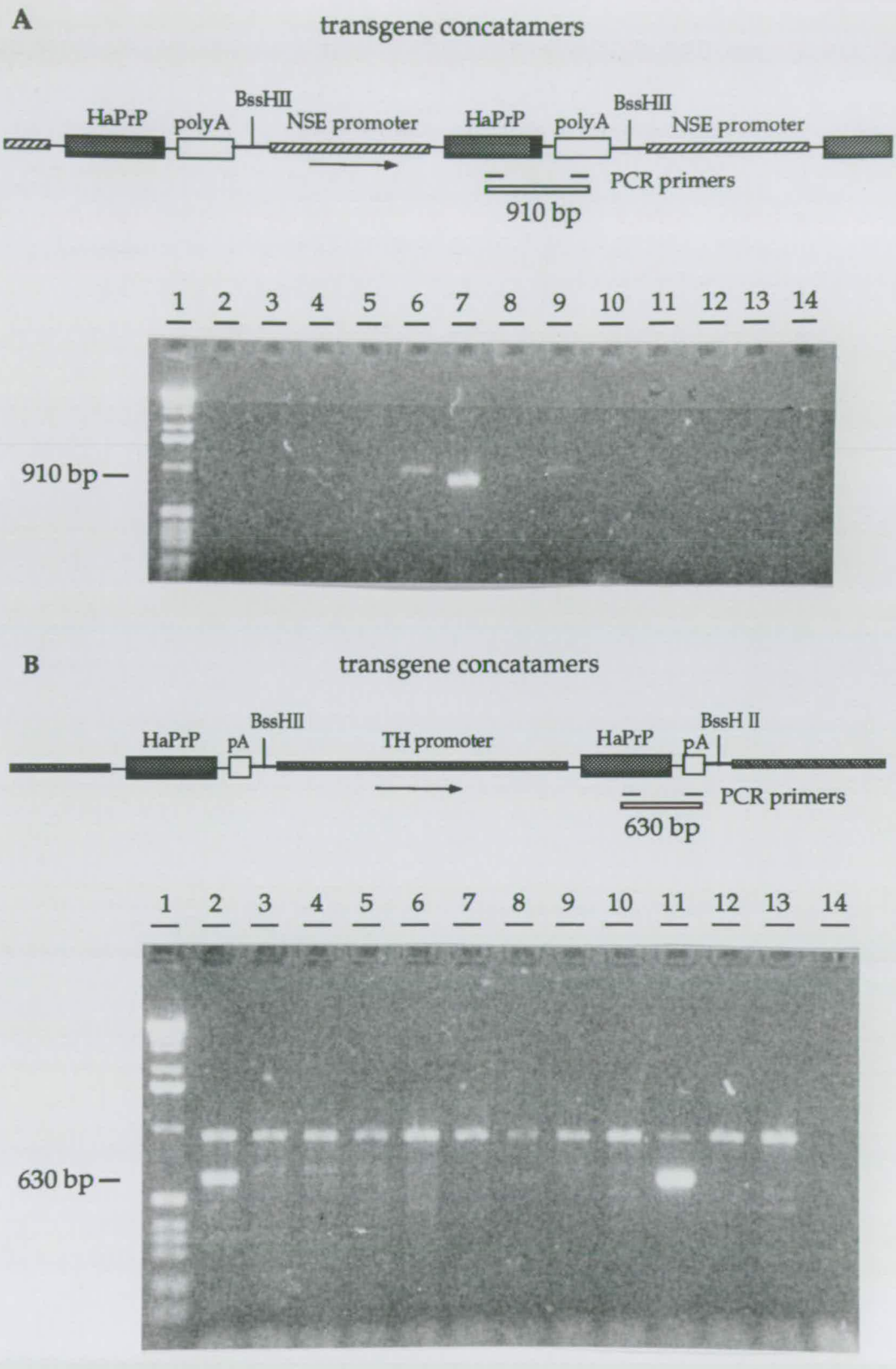

A
transgene concatamers
HaPrP
polyA
BssHII
NSE promoter
HaPrP
polyA
BssHII
NSE promoter
PCR primers
910 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
910 bp
B
transgene concatamers
HaPrP
pA
BssHII
TH promoter
HaPrP
pA
BssH II
PCR primers
630 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14
630 bp

figure 2.4.1

Transgenic offspring generated were analysed for copy number by dot blot and Southern blot analysis. The BssHII fragment used for generation of transgenics (Figure 2.3.6) was used as a copy number control on the dot blots and sometimes on the Southern blots. The amount of transgene fragment used was estimated as described in 7.31.3. Dot blots (examples shown in Figure 2.4.2) were usually probed with fragments containing the SV40 sequence present only in the transgene, so as to avoid the hybridisation signal produced by the endogenous PrP gene. Hybridisation of DNA samples from Tg NSE HaPrP lines gave a higher signal when the EcoRI-BamHI SV40-lacZ fragment (Figure 2.3.1) was used, while the TH HaPrP lines gave better results with the KpnI-XhoI SV40 fragment (Figure 2.3.5), because the probe had greater homology to the respective transgene sequence. The amount of genomic DNA used for the dot blots was variable and therefore, the signal was not always of the same intensity between Tg mice of the same line, sometimes creating problems with copy number estimation and identification of positive Tg mice. For this reason the dot blots were often repeated, or hybridised with different probes and any further ambiguity was resolved by Southern blot analysis. Copy numbers are summarised in Table 2.4.2.

The integrity of the transgene was investigated by Southern blot analysis (Figures 2.4.3 and schematic presentation Figure 2.4.4). Southern blots were hybridised with the 900 bp HindIII-EcoRI HaPrP ORF-containing fragment (Figure 2.3.5). The HaPrP ORF is 90% homologous to the MoPrP ORF and therefore this probe hybridised with the endogenous, single copy, MoPrP gene. Comparison of the intensity of the endogenous MoPrP band with the transgene band would give an approximate estimation of copy number. EcoRI digest of genomic DNA from the Tg NSE HaPrP transgenic lines showed the expected 2.8 kb fragment containing the transgene and a HindIII digest showed the expected 4 kb fragment containing the transgene (schematically presented in Figure 2.4.4A). In the Tg TH HaPrP lines, both EcoRI and HindIII digests revealed the expected 0.9 kb fragment containing the transgene (schematically presented in Figure 2.4.4A). EcoRI digest revealed the expected 2.1 kb fragment and the HindIII digest the expected 2.6 kb fragment of the endogenous MoPrP gene. Control digests of NSE HaPrP poly(A) and TH HaPrP poly(A) BssHII fragments (transgenes)

mixed with non-Tg genomic DNA, usually showed bands of the same size as those obtained from digests of transgenic mouse DNA (transgene concatamers). HindIII digest of the NSE HaPrP poly(A) BssHII transgene fragment showed the expected 2.3 kb fragment (Figure 2.4.3B lane 11) which is different from the 4 kb fragment obtained from the Tg NSE HaPrP genomic DNA due to transgene concatamerisation (see Figure 2.4.4Ab). Fainter bands evident in Tg NSE 20, Tg TH 1, Tg TH 10 and Tg TH 15 at unexpected sizes are probably result of transgene rearrangements and/or interrupted coding sequences, which are not expected to interfere with transgene expression since there are also copies of the transgene that are intact. The restriction patterns were the same for the founder mice and their progeny, the copy numbers were constant and the transgene was transmitted in a Mendelian fashion. These data are consistent with observations suggesting that the transgene is integrated as a tandem "head to tail" array into a single autosomal site (Palmiter and Brinster, 1986) in lines Tg NSE 14 and Tg NSE 17, but aberrant copies could also be present in the rest of the lines.

The results of the generation of transgenic lines are summarised in tables 2.4.1 and 2.4.2.

Table 2.4.1 Generation of transgenic mice

construct	eggs injected	eggs implanted	mice born	transgenic
pNSE HaPrP	252	214	26	4
pTH HaPrP	163	147	16	3

Table 2.4.2 Characterisation of transgenic lines

construct	line	copy number	transmission	integrity
pNSE HaPrP	14	2	+	+
	17	1-2	+	+
	20	15	+	+
	23	1	−	−
pTH HaPrP	1	1	+	+
	10	1-2	+	+
	15	15	+	+

Figure 2.4.2 Dot blot analysis of transgenic mice

Blots A and B were probed with the SV40 EcoRI-BamHI fragment.
Blots C and D were probed with the SV40 KpnI-XhoI fragment.
Each line represents different litters from different generations of Tg mice.

Dot blot A
line 1: litter of Tg NSE 14
line 2: litter of Tg NSE 20
line 3-4: litter of Tg NSE 17
line 5: -
line 6 (6a-6c): control NSE fragment: 10, 5, 0 copies

Dot blot B
line 1: litter of Tg NSE 14
line 2-3: litter of Tg NSE 17
column j : control NSE fragment: 1, 5, 10 copies

Dot blot C
line 1 (1c-1e): control TH fragment 1, 2, 5 copies
line 2: litter of Tg TH 1
line 3-4: litter of Tg TH 10
line 5: litter of Tg TH 1
line 6: litter of Tg TH 15

Dot blot D
line 1-3: litter of Tg TH 1
line 4-5: litter of Tg TH 10
line 6: litter of Tg NSE 14
line 7: litter of Tg NSE 20

For example in dot blot A positive (transgenic) mice were considered those corresponing to the following dots: 1b, 1g, 1i, 1j, 2b, 2c, 2e, 3g, 3h, 3i, 3j.

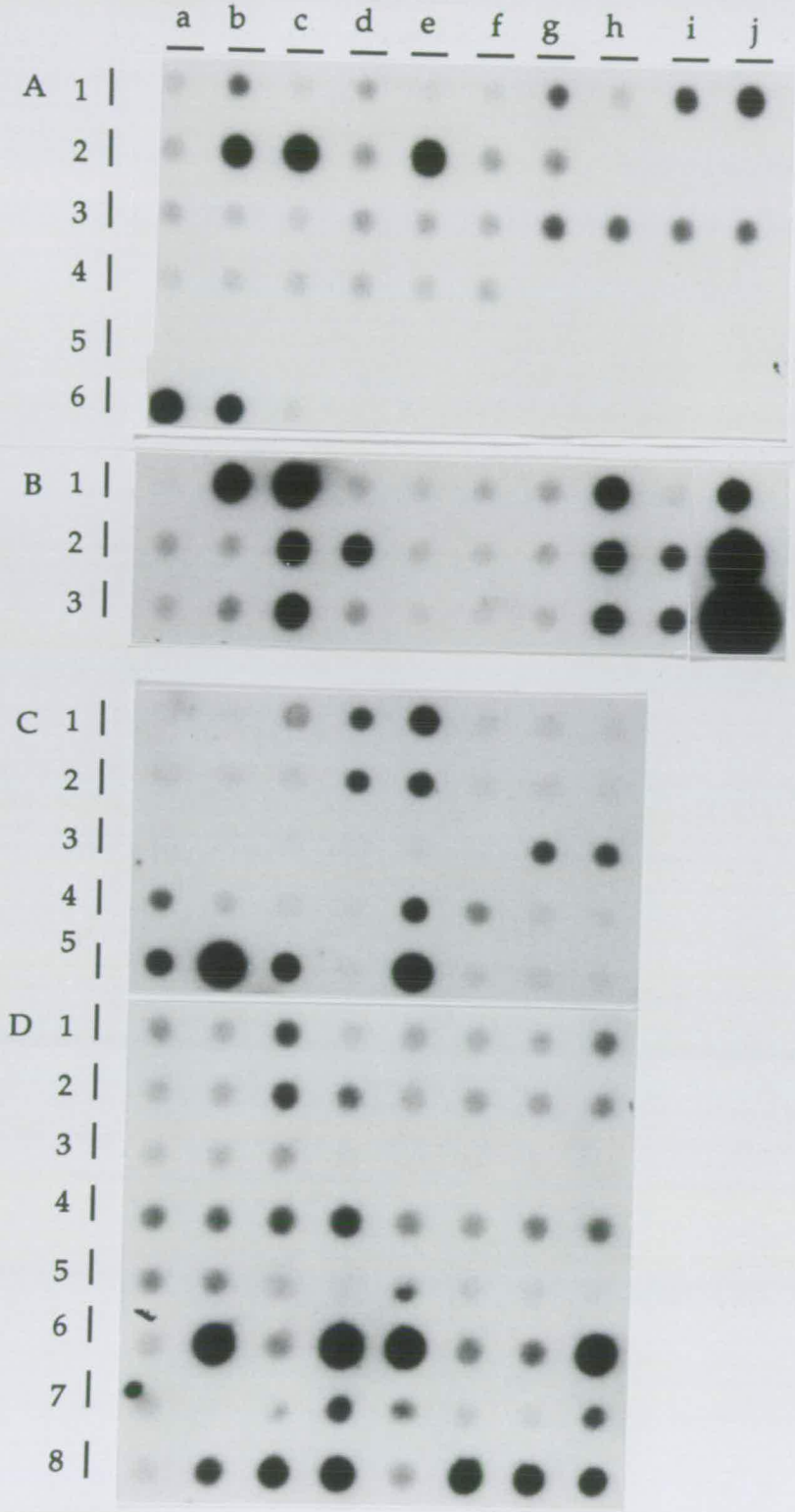


figure 2.4.2

Figure 2.4.3 Southern blot analysis of transgenic mice

A. EcoRI digests of genomic DNA revealed a 2.1 kb fragment corresponing to the endogenous MoPrP, a 2.8 kb NSE-HaPrP transgene-derived fragment (in Tg NSE mice, schematically represented in Figure 2.4.4**A**a) and a 0.9 kb HaPrP transgene-derived fragment (in Tg TH mice, schematically represented in Figure 2.4.4**B**a) after hybridisation with the HaPrP HindIII-EcoRI fragment (see text).

1. 1 kb ladder
2. Tg TH 1 (litter 2)
3. Tg TH 10 (litter 1)
4. Tg TH 1 (litter 3)
5. control TH HaPrP poly(A) transgene fragment, 1 copy (see text)
6. Tg TH 1 (litter 4)
7. non-Tg littermate of Tg TH 1 (litter 4)
8. Tg TH 1 (litter 2)
9. Tg NSE 14 (litter 4)
10. non-Tg littermate of Tg NSE 14 (litter 4)
11. Tg NSE 17 (litter 1)
12. 1 kb ladder

B. HindIII digests of genomic DNA revealed a 2.6 kb fragment corresponing to the endogenous MoPrP, a 4 kb NSE-HaPrP transgene-derived fragment (in Tg NSE mice, schematically represented in Figure 2.4.4**A**b) and a 0.9 kb HaPrP transgene-derived fragment (in Tg TH mice, schematically represented in Figure 2.4.4**B**b) after hybridisation with the HaPrP HindIII-EcoRI fragment (see text).

1. 1 kb ladder
2. non-Tg littermate of Tg TH 10 (litter 3)
3. non-Tg littermate of Tg TH 10 (litter 2)
4. Tg TH 10 (litter 2)
5. non-Tg littermate of Tg TH 15 (litter 2)
6. Tg TH 15 (litter 2)
7. non-Tg littermate of Tg TH 15 (litter 2)
8. control TH HaPrP poly(A) transgene fragment, 1 copy (see text)
9. Tg NSE 20 (litter 4)
10. non-Tg littermate of Tg NSE 20 (litter 4)
11. control NSE HaPrP poly(A) transgene fragment, 1 copy (see text)
12. non-Tg littermate of Tg NSE 20 (litter 4)

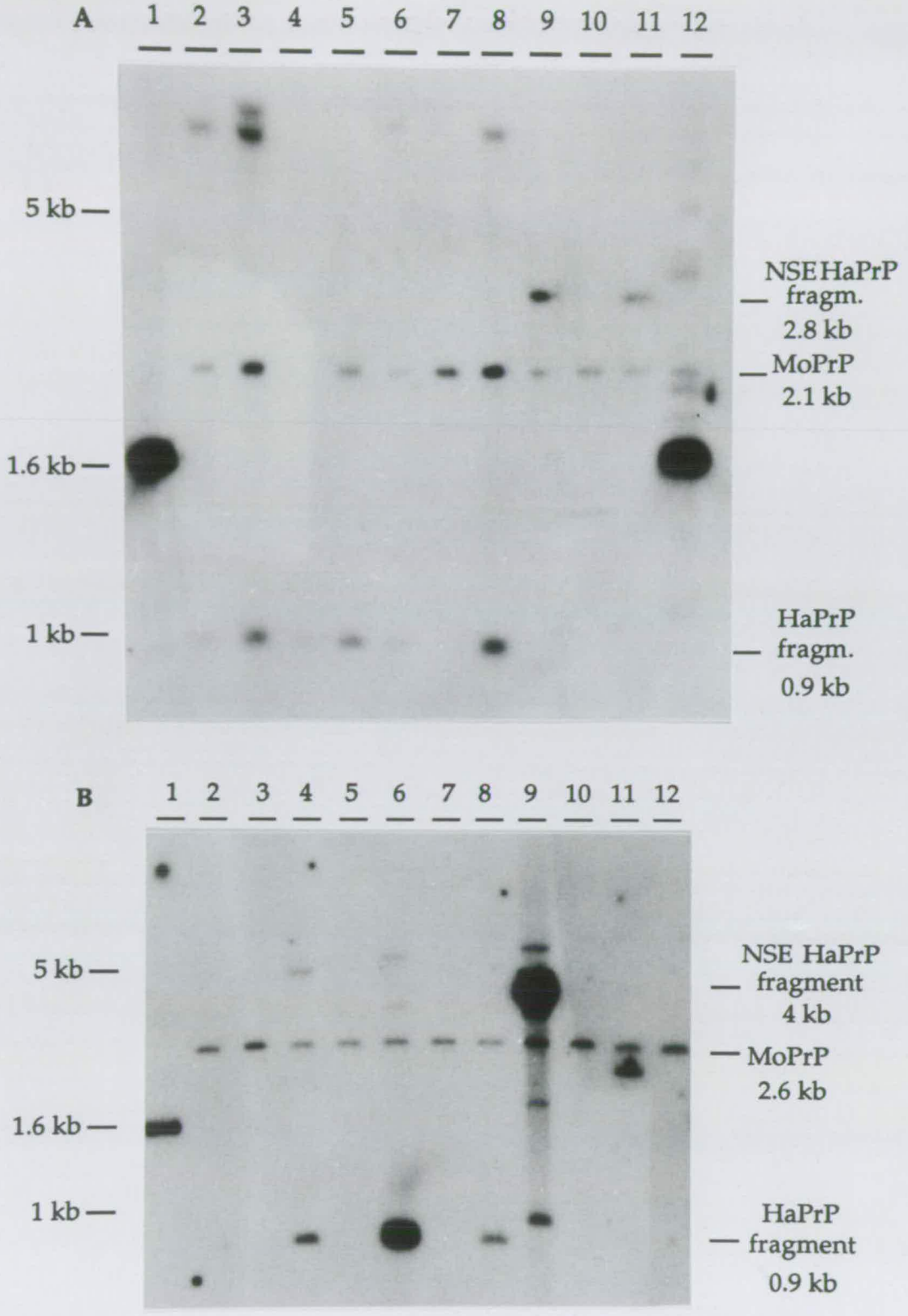

A
1 2 3 4 5 6 7 8 9 10 11 12
5 kb
1.6 kb
1 kb
NSE HaPrP fragm. 2.8 kb
MoPrP 2.1 kb
HaPrP fragm. 0.9 kb
B
1 2 3 4 5 6 7 8 9 10 11 12
5 kb
1.6 kb
1 kb
NSE HaPrP fragment 4 kb
MoPrP 2.6 kb
HaPrP fragment 0.9 kb

figure 2.4.3

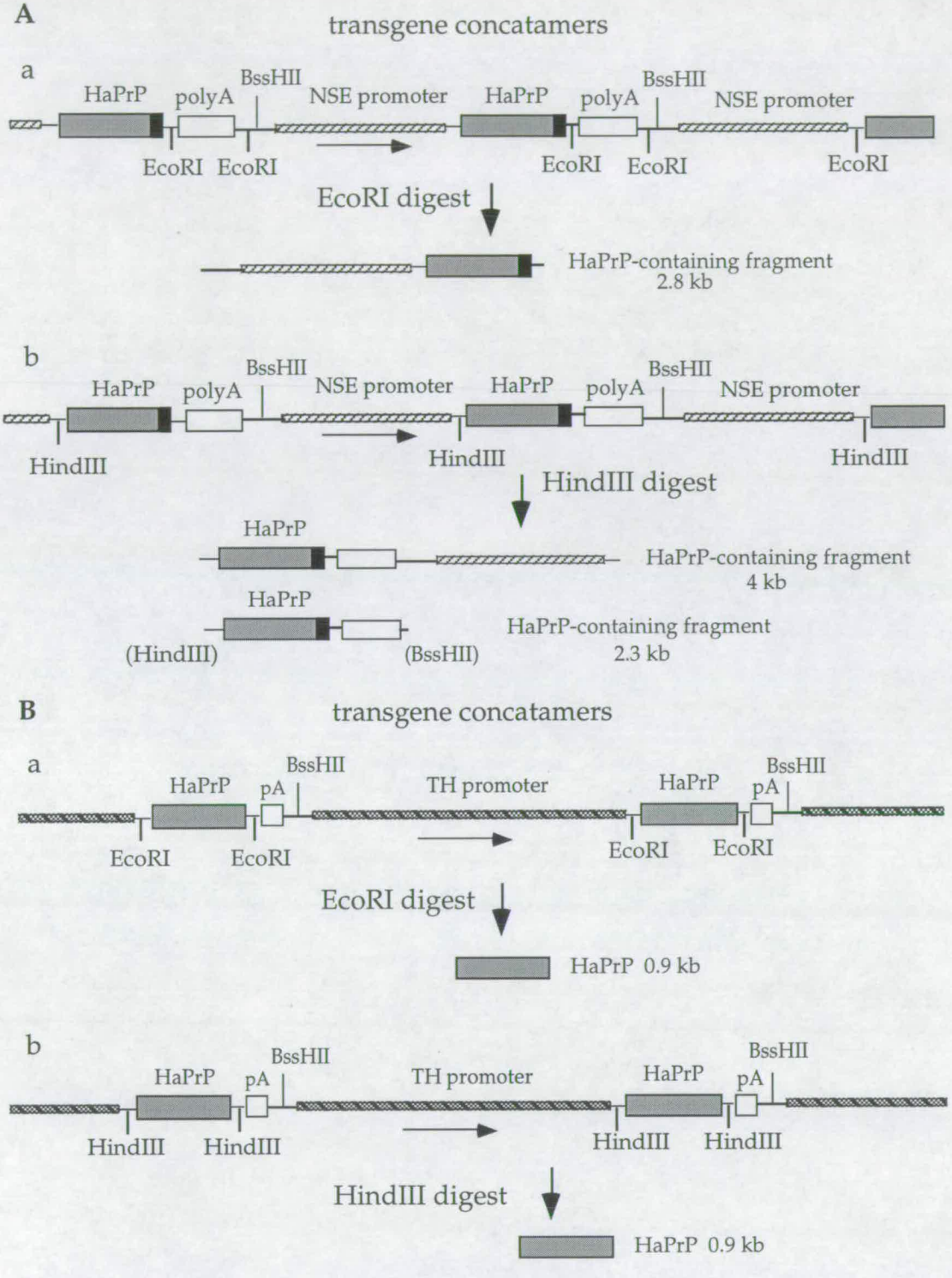


Figure 2.4.4 Schematic presentation of EcoRI and HindIII digests of genomic DNA from Tg NSE and Tg TH mice

2.5 Expression of transgene in Tg NSE HaPrP lines

The Tg NSE HaPrP lines were analysed for transgene expression by Northern blot analysis (sections 7.16 and 7.18). Total RNA was isolated from various tissues of Tg and non-Tg littermates (section 7.8). Hybridisation of brain RNA with the HaPrP ORF fragment revealed a 2.3 kb mRNA species corresponding to the endogenous MoPrP transcript and a 1.7 kb mRNA species corresponding to the transgene mRNA (Figure 2.5.1A and 2.5.1C). Similar results were obtained when the MoPrP ORF was used as a hybridisation probe (Figure 2.5.1D). When the SV40 poly(A) fragment was used as a probe only the mRNA produced by the transgene gave a hybridisation signal, although some faint bands, probably background hybridisation, were observed in all brain samples (Figure 2.5.1B). Phosphorimager detection and quantification analysis (Molecular Dynamics software package), was used to estimate the levels of mRNA. Total hamster brain RNA was used as a positive control (Figure 2.5.1C) and other mouse tissues were included in the analysis (Figures 2.5.1A,B,D). In the highest expressing line, Tg NSE 20, the expression of the transgene was approximately 15% of that of the endogenous MoPrP gene. Tg NSE 14 showed approximately 25% expression in comparison to Tg NSE 20 (2-5% in comparison to the endogenous). Tg NSE 17 did not show any expression of the transgene which could be due to the site of integration, or methylation state of the transgene. Endogenous MoPrP mRNA was detected in other tissues apart from brain (see also section 1.6). Quantification of PrP mRNA from various tissues, normalised against ubiquitously expressed ribosomal protein (S26) mRNA revealed that heart PrP mRNA was approximately 25-30%, lung 25%, kidney 15%, spleen 10%, muscle 10% and liver 0-1% in comparison to brain PrP mRNA. However, HaPrP mRNA produced from the line Tg NSE 20 transgene was present only in the brain (Figure 2.5.1A,B,D).

2.6 Expression of HaPrP protein in Tg NSE HaPrP lines

To study PrP protein expression, several tissues from Tg NSE 20 and Tg NSE 14 mice and non-Tg littermates, as well as hamster brain

Figure 2.5.1 Northern blot analysis of Tg NSE HaPrP lines

A. Probed with HaPrP ORF fragment.

B. Probed with EcoRI -BamHI SV40 fragment.

The same samples in the same order were used for blots **A** and **B**

1-5 brain samples

1. Tg NSE 20 (litter 3)
2. Tg NSE 14 (litter 2)
3. Tg NSE 17 (litter 1)
4. non-Tg littermate of Tg NSE 20 (litter 3)
5. non-Tg littermate of Tg NSE 14 (litter 2)
6. Tg NSE 20 spleen (litter 3)
7. Tg NSE 20 liver (litter 3)

C. Probed with HaPrP ORF fragment.

1. hamster brain
2. hamster brain 1:10 dilution of sample 1 in sterile water
3. hamster brain 1:20 dilution of sample 1 in sterile water
4. Tg NSE 20 brain (litter 4)
5. Tg NSE 20 brain (litter 4) 1:10 dilution in sterile water
6. Tg NSE 20 brain (litter 4) 1:20 dilution in sterile water

(samples 2 and 3 were partially degraded)

D. Probed with MoPrP ORF fragment.

Control hybridisation with S26 (mouse ribosomal protein coding fragment) is also presented.

1. F1 mouse brain
2. F1 mouse heart
3. F1 mouse liver
4. F1 mouse lung
5. Tg NSE 20 (litter 5) brain
6. Tg NSE 20 (litter 5) heart
7. Tg NSE 20 (litter 5) muscle
8. Tg NSE 20 (litter 5) liver
9. Tg NSE 20 (litter 5) spleen
10. Tg NSE 20 (litter 5) kidney

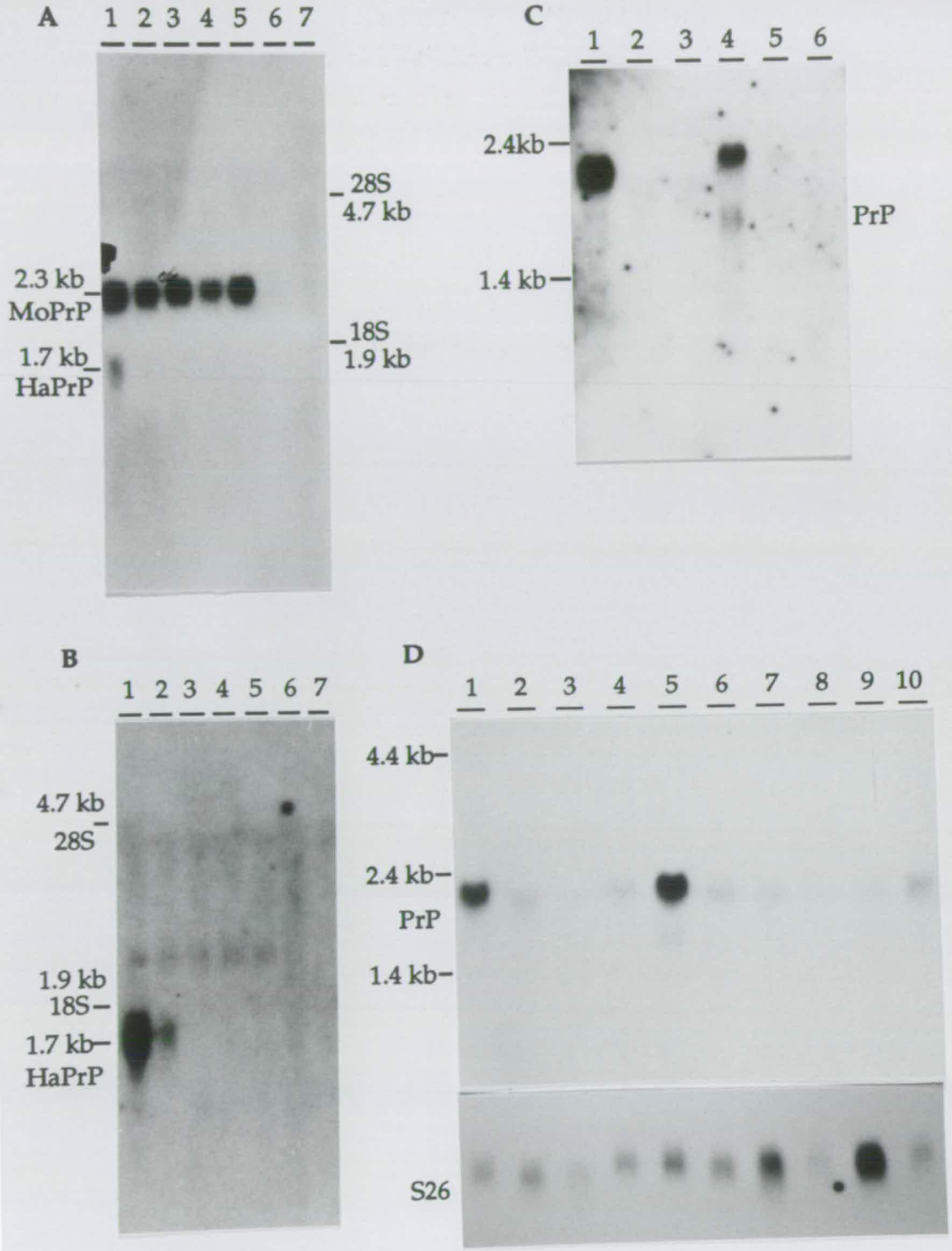

A
1 2 3 4 5 6 7
28S
4.7 kb
2.3 kb
MoPrP
18S
1.9 kb
1.7 kb
HaPrP
C
1 2 3 4 5 6
2.4kb
1.4 kb
PrP
B
1 2 3 4 5 6 7
4.7 kb
28S
1.9 kb
18S
1.7 kb
HaPrP
D
1 2 3 4 5 6 7 8 9 10
4.4 kb
2.4 kb
PrP
1.4 kb
S26

figure 2.5.1

protein samples, were analysed by Western immunoblotting (section 7.21.6).

Brain protein samples from Tg NSE HuPrP line 42 (one of the Tg lines carrying the HuPrP ORF generated by Dr. P. Estibeiro and Dr. O. Windl), shown to express HuPrP mRNA in the brain (Dr. P. Estibeiro and Dr. O. Windl personal communication) were included in the analysis. The antibodies (ab) used were the monoclonal 3F4 (Kascsak *et al.*, 1987), which reacts with hamster and human, but not mouse PrP and the polyclonal 1B3 and 1A8, which react with PrP produced from different species, including mouse hamster and man (Farquhar *et al.*, 1989). From all samples tested, only the endogenous mouse or hamster PrP protein was detected in the brain samples (Figures 2.6.1 and 2.6.2).

Endogenous PrP protein can be detected in other tissues apart from brain, but larger amounts of tissue and more sensitive detection methods are necessary in order to achieve this (Horiuchi *et al.*, 1995). To test the sensitivity of the method used here, serial dilutions of hamster brain protein were used (Figure 2.6.2B,C). It was revealed that PrP protein could be detected in samples containing 5% (1:20 dilution) of the amount usually used for immunoblotting (approximately 20 μg). This result indicated that if the mRNA produced by the transgene was translated as efficiently as the endogenous PrP message, the protein present in Tg NSE line 20 and possibly also line 14, should be detectable (mRNA levels 15% and 4% of the endogenous MoPrP mRNA levels).

No HaPrP protein was detected in any brain samples from Tg NSE HaPrP lines. Similar results were obtained with Tg mice generated with a transgene carrying the HuPrP ORF (Figures 2.6.1A,B, 2.6.2A and Dr. P. Estibeiro and Dr. O. Windl personal communication). Furthermore, these experiments provided clear evidence for downregulation of endogenous PrP translation in other tissues compared to brain.

Bands present at lower molecular weight than PrP (approximately 16 kD - 19 kD) are sometimes present in some samples (see Figure 2.6.1C 2.6.2 and 4.6.10). These bands are not transgene-related because they are also present in samples from non-Tg mice.

Figure 2.6.1 Western blot assay of Tg NSE HaPrP mice

A. Probed with the mouse monoclonal ab 3F4 (hamster specific).

B. Probed with the rabbit polyclonal anti-PrP ab 1B3.

The same samples in the same order were used for **A** and **B**.

1. size markers
2. hamster brain
3. Tg NSE 20 brain (litter 2)
4. Tg NSE 14 brain (litter 2)
5. control non-Tg mouse brain
6. Tg NSE 42 brain (litter 2) carrying the HuPrP ORF
7. Tg NSE 20 lung (litter 2)
8. Tg NSE 20 kidney (litter 2)

C. Probed with the mouse monoclonal ab 3F4 (hamster specific).

1. size markers
2. control non-Tg mouse brain
3. Tg NSE 20 brain (litter 3)
4. Tg NSE 20 liver (litter 3)
5. Tg NSE 20 spleen (litter 3)
6. hamster brain
7. scrapie-infected hamster brain
8. purified hamster PrP

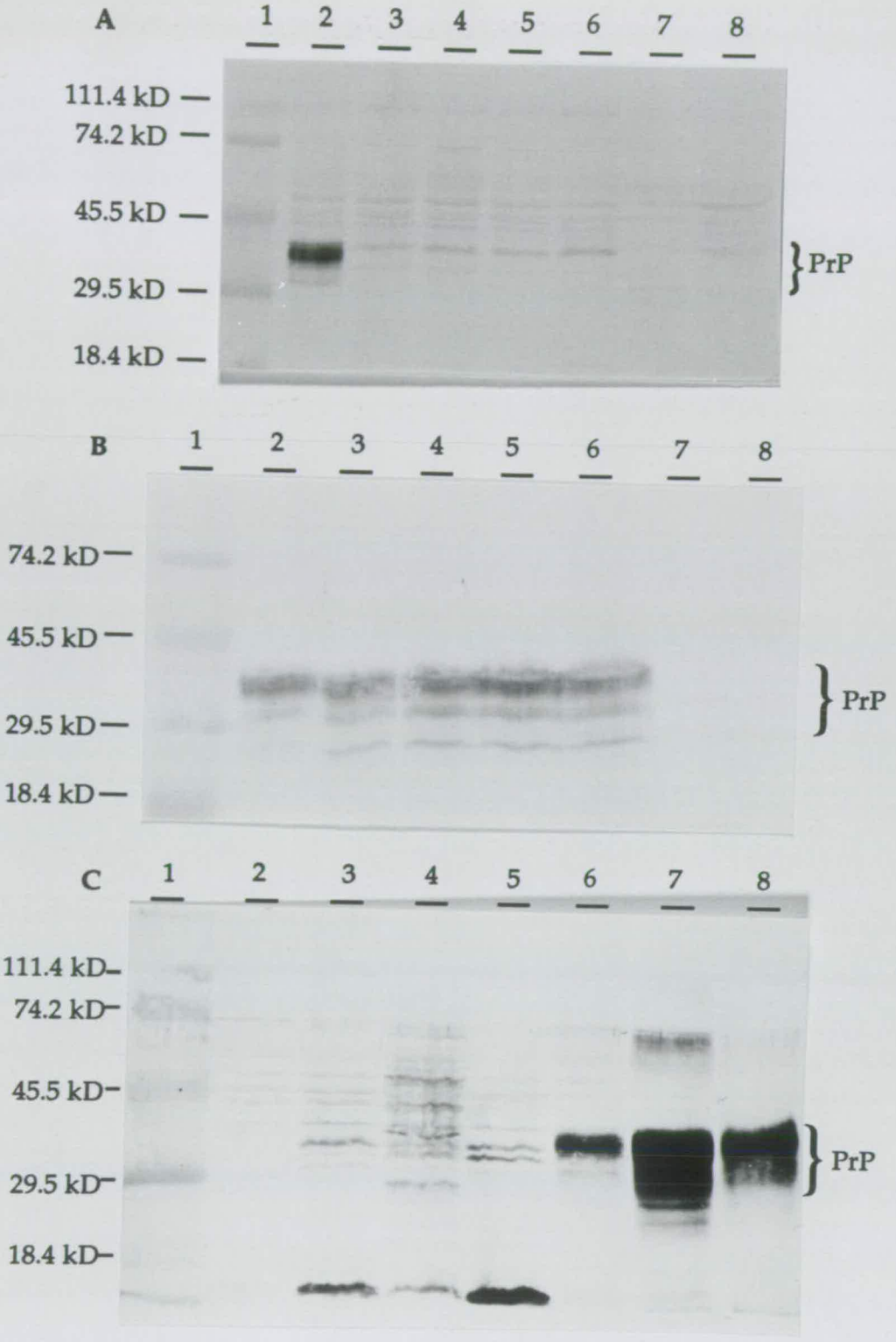

A
1 2 3 4 5 6 7 8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
B
1 2 3 4 5 6 7 8
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1 2 3 4 5 6 7 8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 2.6.1

Figure 2.6.2 Western blot assay of Tg NSE HaPrP mice

A. and **B**. Probed with the mouse monoclonal ab 3F4 (hamster specific).
C. Probed with the rabbit polyclonal anti-PrP ab 1A8.

A.

1. size markers
2. hamster brain
3. Tg NSE 20 brain (litter 4)
4. Tg NSE 14 brain (litter 3)
5. F1 mouse brain
6. Tg NSE 42 brain (litter 3) carrying the HuPrP ORF
7. Tg NSE 14 heart (litter 3)
8. Tg NSE 20 kidney (litter 3)

B and **C**. The same samples were used in the same order.

1. hamster brain
2. hamster brain 1:10 dilution
3. hamster brain 1:20 dilution
4. Tg NSE 20 brain (litter 4)
5. F1 mouse brain
6. Tg NSE 20 heart (litter 4)
7. Tg NSE 20 lung (litter 4)
8. Tg NSE 20 spleen (litter 4)

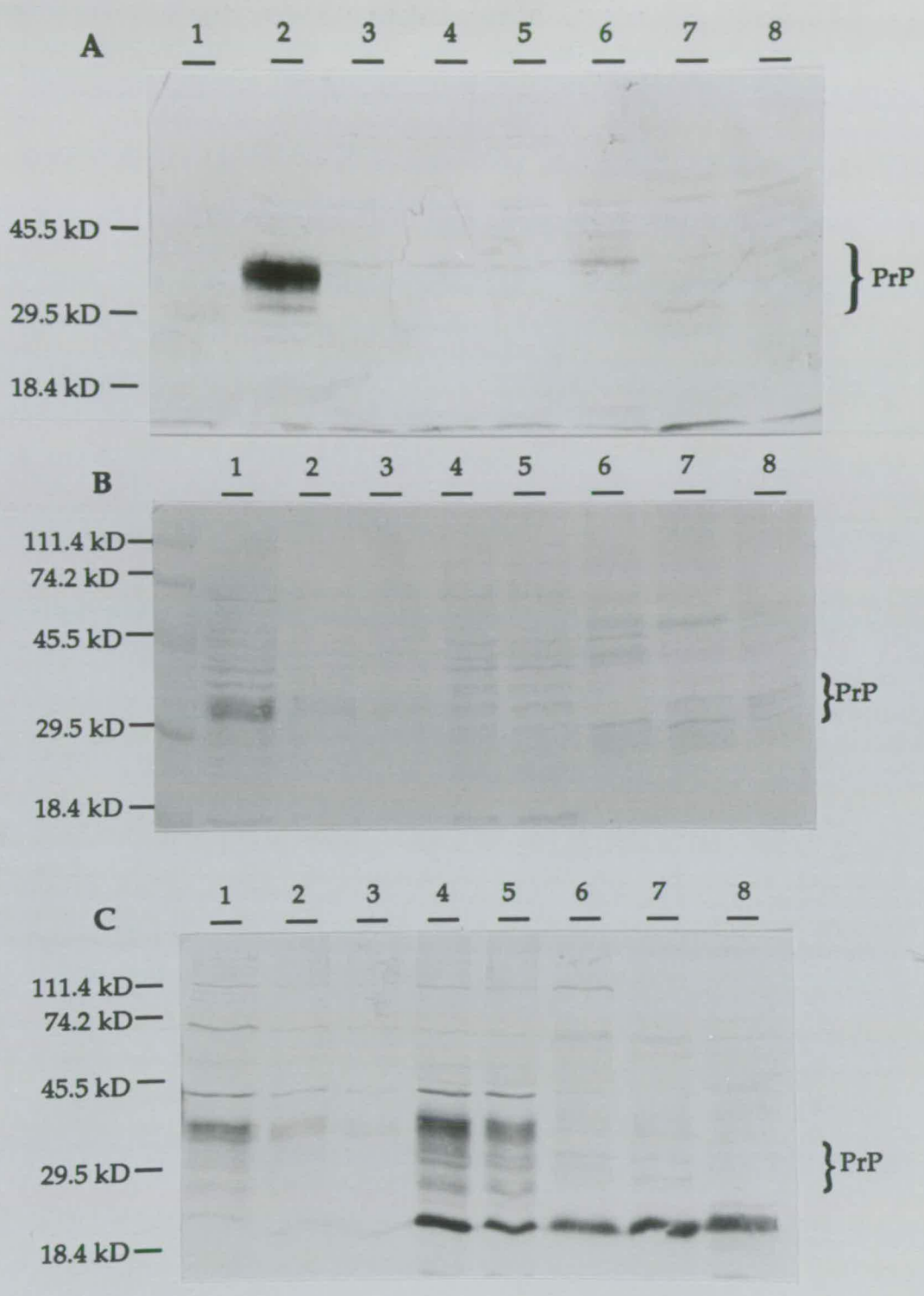

A
1
2
3
4
5
6
7
8
45.5 kD
29.5 kD
18.4 kD
PrP
B
1
2
3
4
5
6
7
8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1
2
3
4
5
6
7
8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 2.6.2

2.7 Expression of transgene in Tg TH HaPrP lines

In situ hybridisation (section 7.32) was performed to determine the localisation of the mRNA produced by the TH HaPrP transgene. Northern blot analysis and reverse transcription PCR (RT-PCR section 7.6.12) on dissected brain regions were also performed.

Several attempts were made to detect HaPrP mRNA by different *in situ* hybridisation protocols (sections 7.32.3 and 7.32.4). Three anti-sense oligos were initially chosen as probes (section 7.17.3) and the equivalent sense oligos were used as control probes. The oligo SV40-a (appendix 1) was located in the SV40 sequence directly downstream of the XhoI site in pTH HaPrP (Figure 2.3.5) and is therefore unique to the transgene sequences (SV40-s oligo is the sense equivalent). The other two oligos were located in the MoPrP ORF (Mo-a, appendix 1) and in the HaPrP ORF (Ha-a, appendix 1) in a region 50-100 nt upstream of the TGA stop codon that is 25% different between the two species. The Ha-a probe should hybridise only to HaPrP and the Mo-a only to the endogenous MoPrP (Mos and Ha-s were the sense oligos used, see appendix 1). A tyrosine hydroxylase-derived oligo, TH-a (appendix 1) was used as a control for endogenous TH expression.

When tested on normal mouse brain (coronal sections of frozen brain) the Ha-a was expected to give no signal and the Mo-a was expected to hybridise to the endogenous MoPrP gene. However both probes gave a signal, but the hybridisation pattern was different between them (Figure 2.7.1). The Ha-a probe gave hybridisation signal mainly in the corpus callosum, the cingulum, the dentate gyrus of the hippocampus, the lateral geniculate nuclei and the lateral and dorsal thalamic nuclei, while the Mo-a probe showed endogenous MoPrP expression mainly in the CA1, CA2, CA3 and dentate gyrus of the hippocampus, cerebral cortex, mammilary nuclei, amygdaloid nuclei, cerebellum and areas in the thalamus and hypothalamus. This finding suggested that the Ha-a probe was cross-hybridising to a mouse RNA (not MoPrP mRNA) and therefore was not useful for detecting transgene-produced HaPrP mRNA.

Hybridisation signal was detected in the olfactory bulb and possibly the ventral tegmental area and the substantia nigra when the SV40-a probe (transgene-specific) was used on the Tg TH HaPrP brain sections

(horizontal sections of frozen brain, Figure 2.7.2). The hybridisation pattern was similar to that obtained by the TH-a probe in the olfactory bulb. Hybridisation signal was detected in the preoptic nuclei, olfactory tubercle, lateral olfactory tract and the olfactory nuclei. Hybridisation in these regions with the SV-a probe was much lower than that observed with the TH-a probe. In the ventral tegmental area and substantia nigra (Figure 2.7.3) the expression pattern obtained with the SV-a probe did not exactly resemble that observed with the TH-a probe. This finding indicated that the signal detected in that region could be due to background hybridisation. The SV40-s probe that was used as a negative control (Figure 2.7.2) did not show any hybridisation in that region, but hybridisation signal was detected in the cochlear nuclei and the spinal trigeminal nuclei. This suggested that the signal observed in these areas with the SV40-a, and TH-a probes was unspecific hybridisation. The Mo-a oligo was used as a positive control showing endogenous MoPrP (Figure 2.7.3).

Alternatively, a riboprobe (section 7.17.4) containing SV40 sequences present in the transgene construct pTH HaPrP (KpnI-XhoI SV40 sequence, Figure 2.3.5) was used to detect transgene expression and the sense strand was used as a negative control (Figure 2.7.4). These probes were used on coronal paraffin embedded brain sections (section 7.32.1). The MoPrP antisense riboprobe (MoPrP ORF derived, provided by the BBSRC Neuropathogenesis Unit, Edinburgh) was used as a positive control, showing endogenous MoPrP expression and the MoPrP sense riboprobe was used as a negative control (Figure 2.7.4). No transgene specific expression was detected, although the background produced with the riboprobes was high, not allowing detection of possible subtle differences.

Using Northern blot analysis on dissected brain regions, a very faint signal at approximately 1.4 kb was detected with the SV40 KpnI-XhoI probe (Figure 2.3.5) in the olfactory bulb (Figure 2.7.5). The intensity of the band was only 5-10% of that present in a Tg NSE line 20 brain sample. RT-PCR was also performed several times on dissected regions of Tg TH HaPrP mouse brain. A band specific for transgene produced mRNA was detected in the olfactory bulb by RT-PCR, although it was also present, but much fainter, in the rest of brain and cortex (Figure 2.7.5) and sometimes in other brain regions (data not shown). This could

Figure 2.7.1 *In situ* hybridisation of coronal cryostat sections of F1 mouse brain with 4 antisense oligo probes.

a. Hybridisation with Mo-a oligo.

b. Hybridisation with Ha-a oligo.

c. Hybridisation with muscarinic receptor 1 derived oligo (gift from Dr M. Steel) revealed mainly hippocapus specific expression (used as a positive control).

d. Hybridisation with opsin derived oligo (gift from Dr M. Steel) gave no signal because opsin is not expressed in the brain (used here as a negative control).

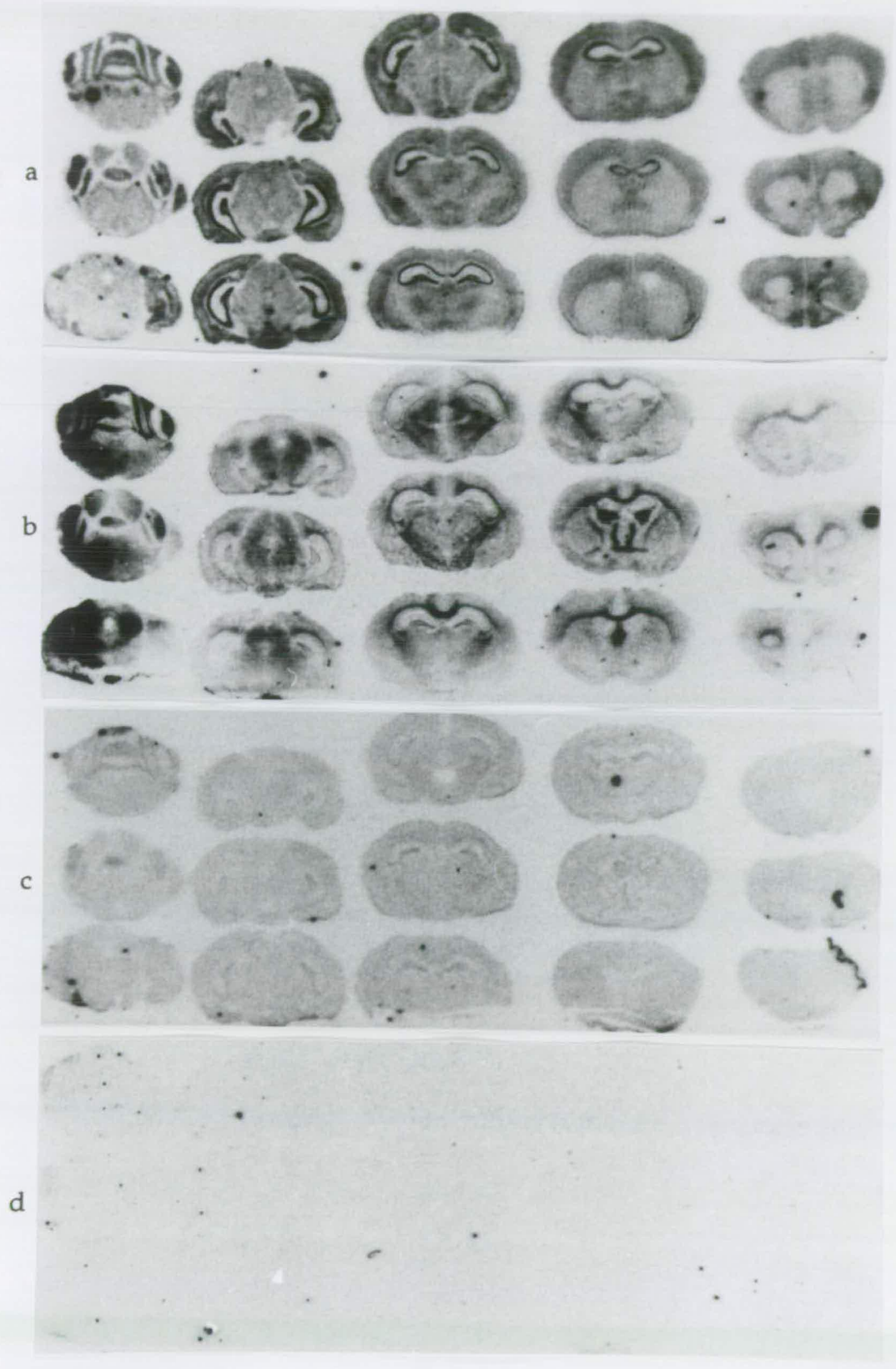
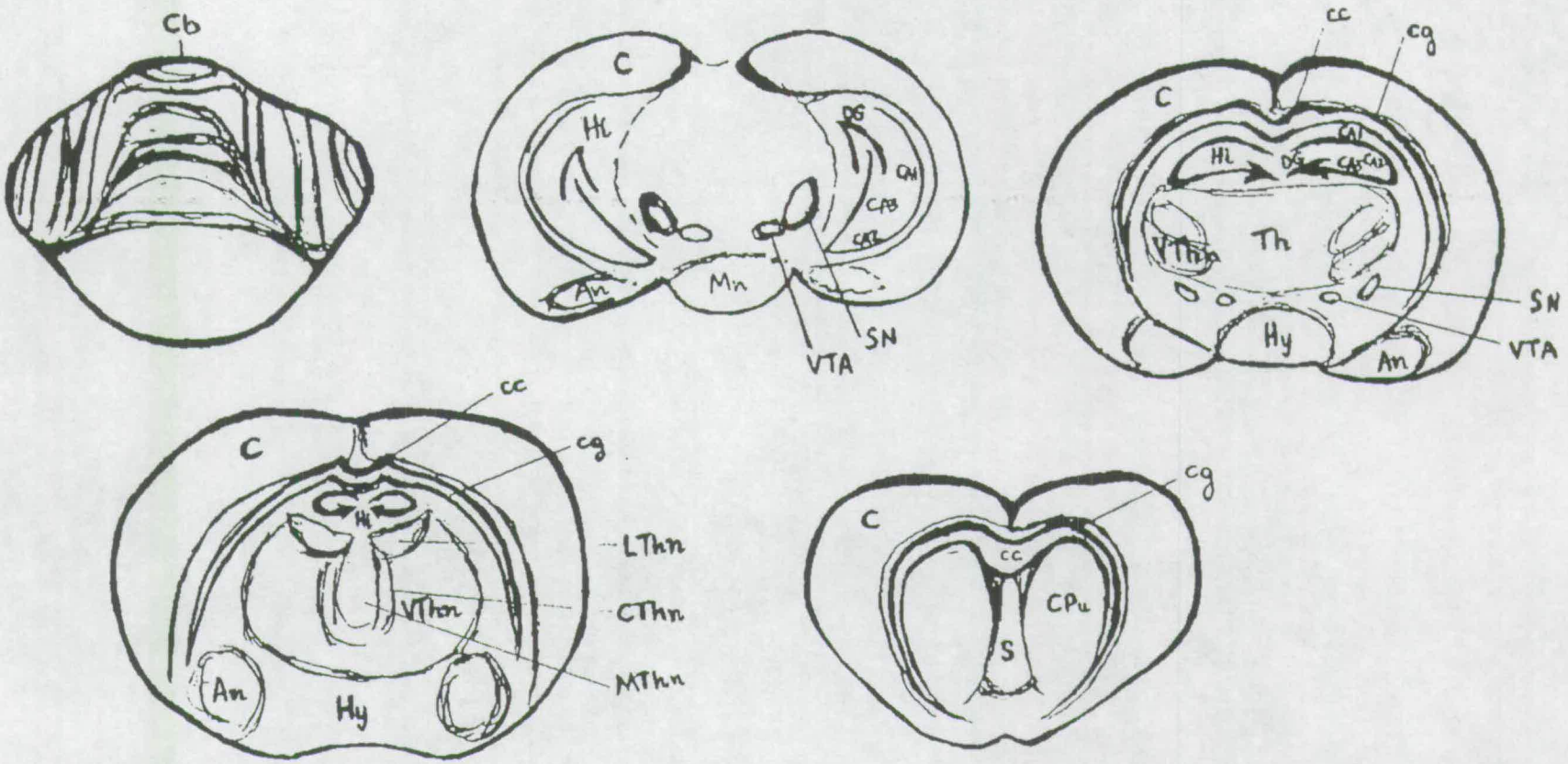


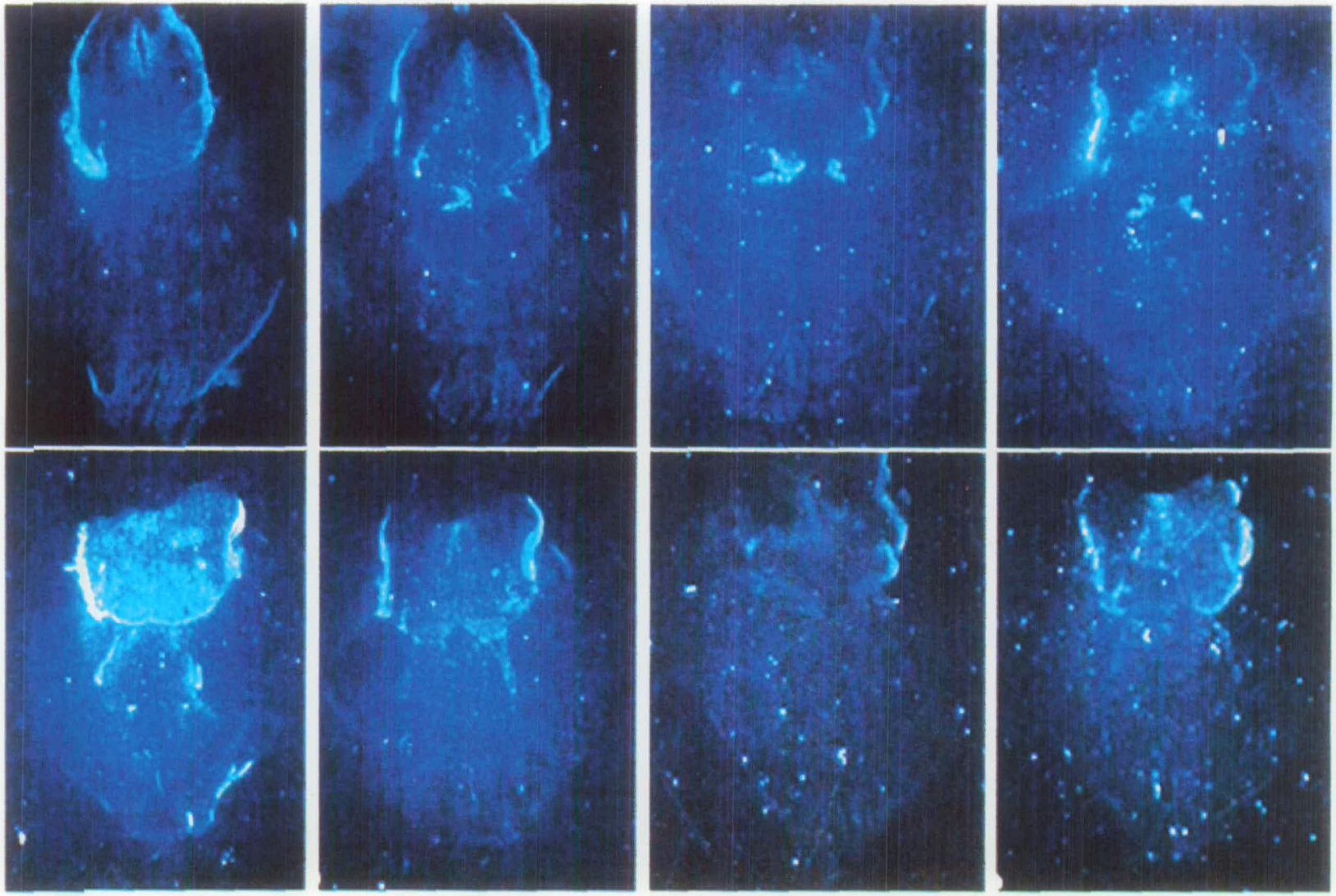
figure 2.7.1

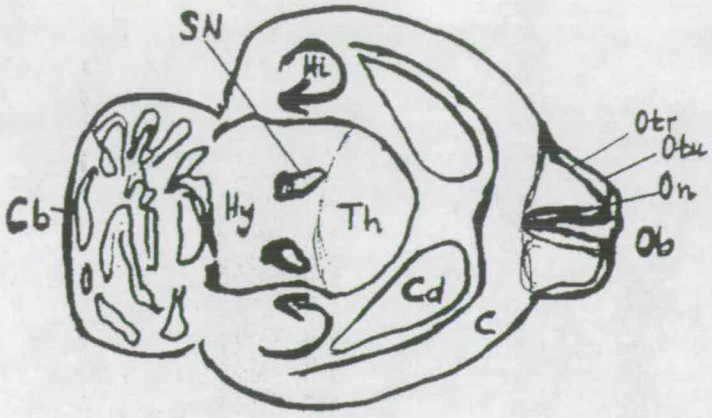


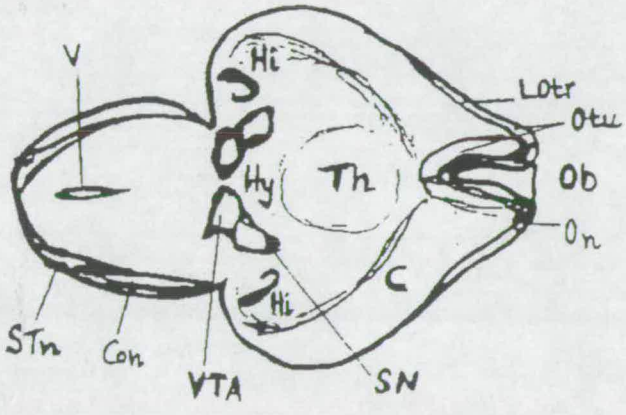
An: amygdaloid nuclei, **C**: cortex, **CA1**, **CA2**, **CA3** regions of the hippocampus, **cg**: cingulum, **Cd**: caudate nucleus, **CPu**: caudate putamen, **Cb**: cerebellum, **cc**: corpus calosum, **CThn**: central thalamic nuclei, **DG**: dentate gyrus (hippocampus), **Hi**: hippocampus, **Hy**: hypothalamus, **LThn**: lateral thalamic nuclei, **Mn**: mammilary nuclei, **MThn**: medial thalamic nuclei, **S**: septum, **SN**: substantia nigra, **Th**: thalamus, **VTA**: ventral tegmental area, **VThn**: ventral thalamic nuclei

Figure 2.7.2 *In situ* hybridisation of horizontal cryostat sections of Tg TH 15 mouse brain with 2 antisense oligo probes.

The 4 pictures on each column are hybridised with the same probe.
The 2 pictures on each line represent adjacent brain sections
column 1. Hybridisation with TH-a oligo, showing endogenous TH expression.
column 2. Hybridisation with SV40-a oligo, showing transgene expression.







C: cortex, Cd: caudate nucleus, Cb: cerebellum, Con: cochlear nuclei, Hi: hippocampus, Hy: hypothalamus, LOtr: lateral olfactory tract, Ob: olfactory bulb, On: olfactory nuclei, Otu: olfactory tubercule, Otr: olfactory tract, STn: spinal trigeminal nuclei, SN: substantia nigra, Th: thalamus, V: ventricle, VTA: ventral tegmental area

Figure 2.7.3 *In situ* hybridisation of horizontal cryostat sections of Tg TH 15 mouse brain with 2 antisense oligo probes.

The 4 pictures on each column are hybridised with the same probe.
The 2 pictures on each line represent adjacent brain sections
column 1. Hybridisation with Mo-a oligo, showing endogenous MoPrP expression.
column 2. Hybridisation with SV40-s oligo, showing background hybridisation (negative control).

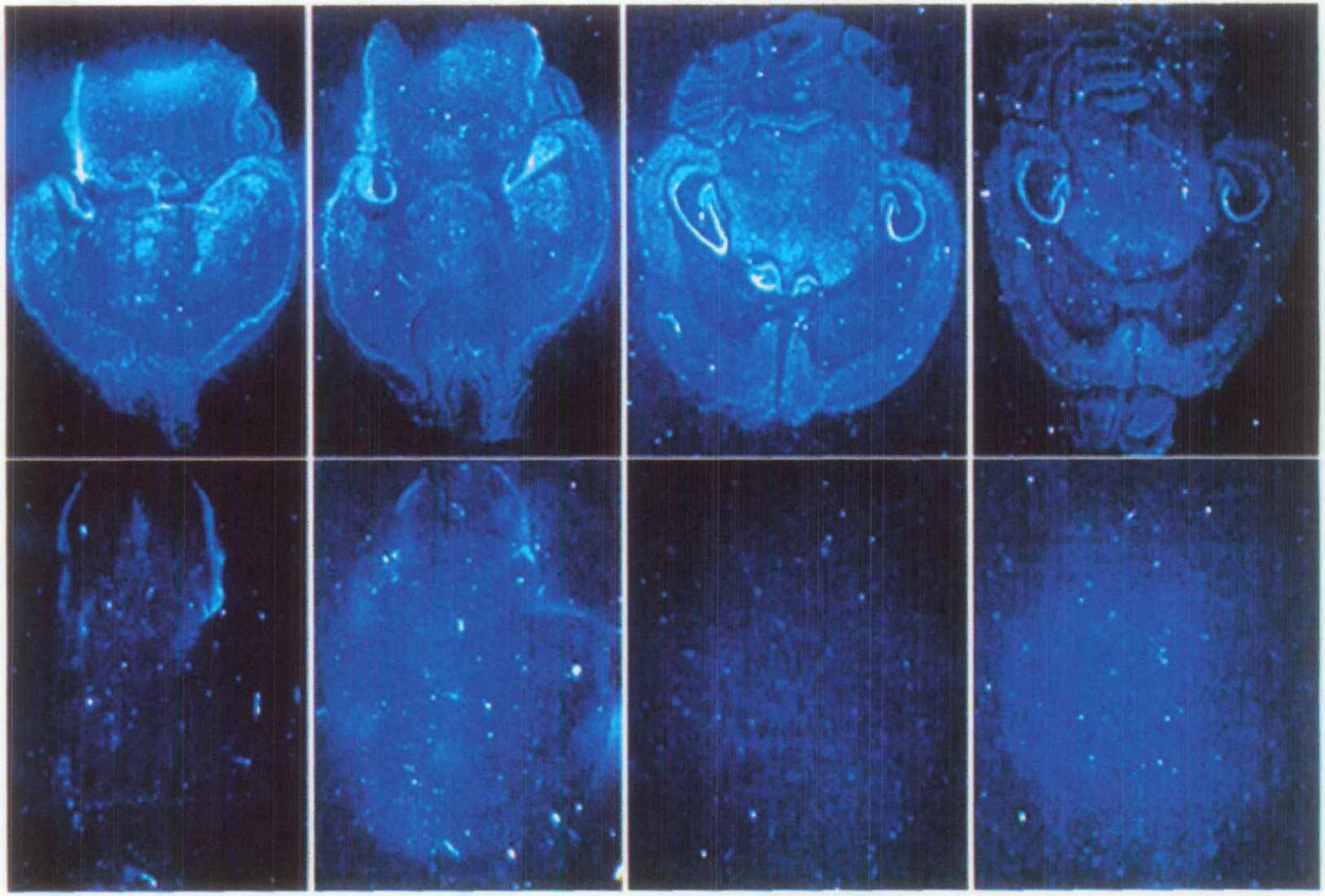


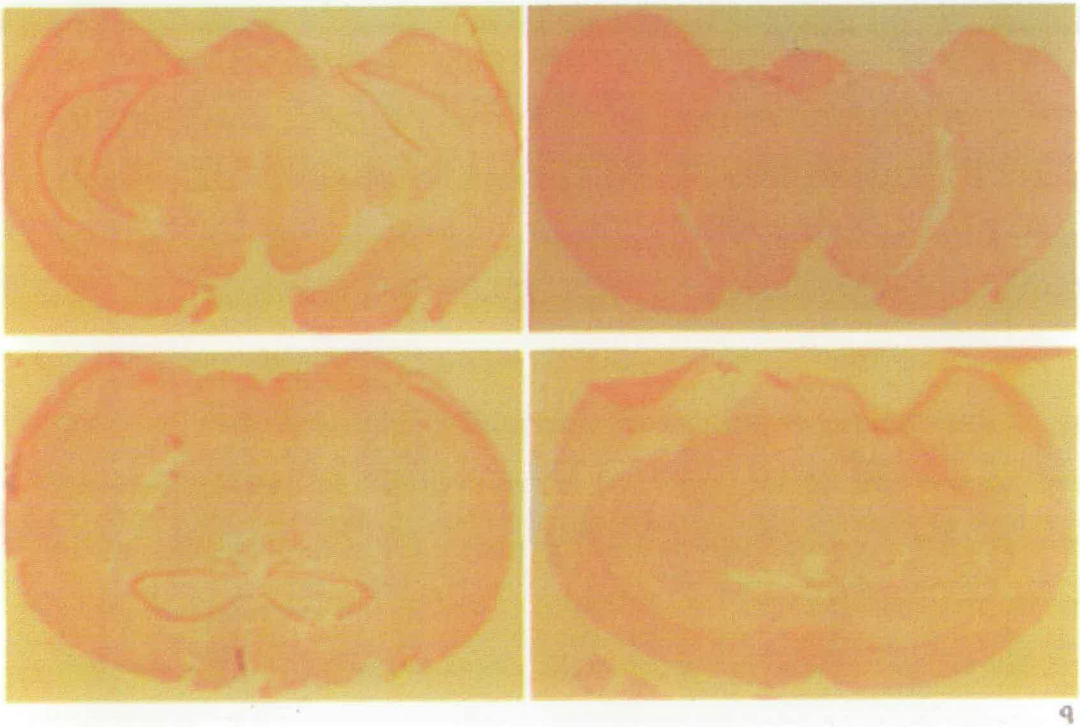
Figure 2.7.4 *In situ* hybridisation of coronal sections of Tg TH 15 paraffin embedded mouse brain with 4 riboprobes.

a.

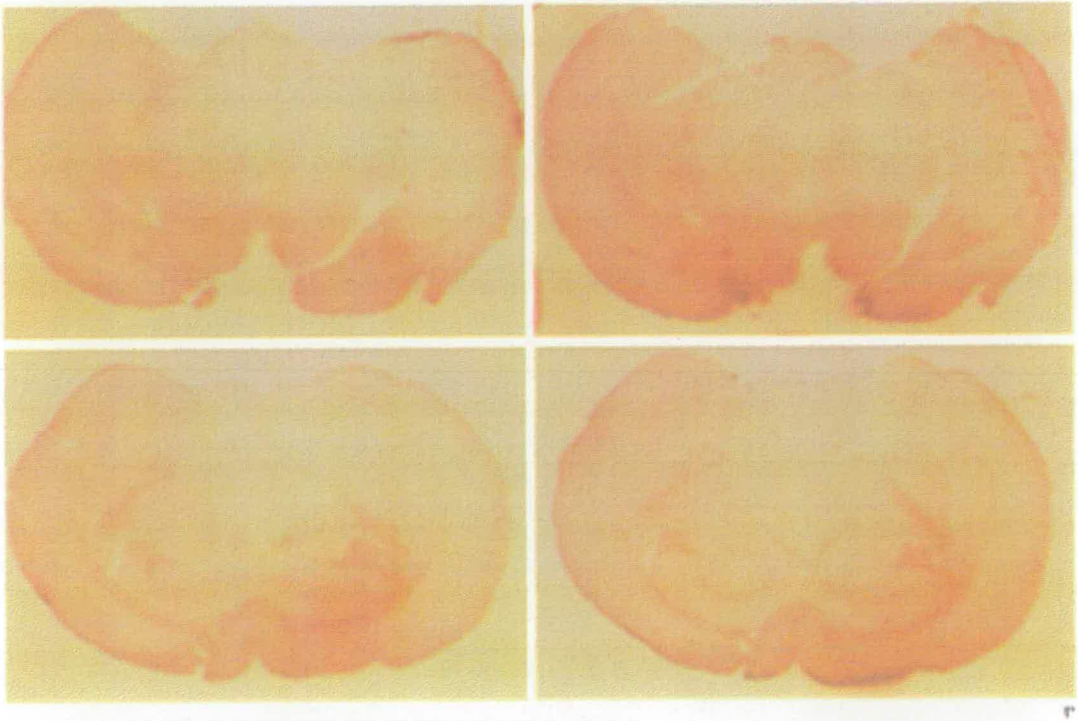
The 2 pictures on each column are hybridised with the same probe.
The 2 pictures on each line represent adjacent brain sections
column 1. Hybridisation with SV40 sense riboprobe, showing background hybridisation (negative control).
column 2. Hybridisation with SV40 antisense riboprobe, showing transgene expression.

b.

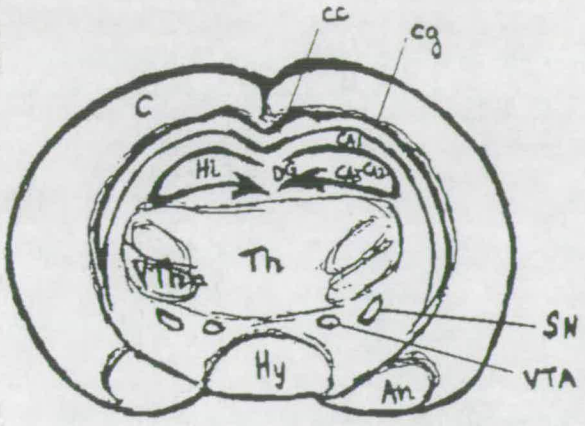
The 2 pictures on each column are hybridised with the same probe.
The 2 pictures on each line represent adjacent brain sections
column 1. Hybridisation with MoPrP sense riboprobe, showing background hybridisation (negative control).
column 2. Hybridisation with MoPrP antisense riboprobe, showing endogenous MoPrP expression.

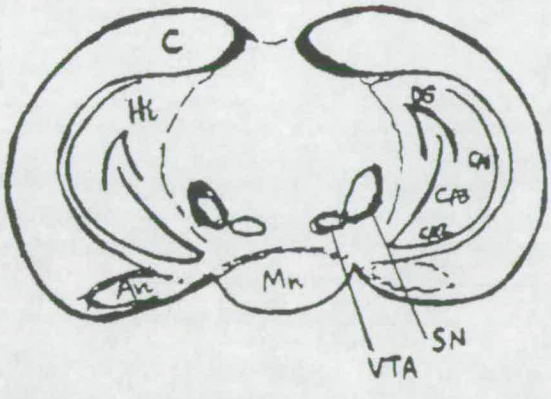


٩



٣





An: amygdaloid nuclei, **C**: cortex, **CA1, CA2, CA3** regions of the hippocampus, **cg**: cingulum, **cc**: corpus calosum, **DG**: dentate gyrus (hippocampus), **Hi**: hippocampus, **Hy**: hypothalamus, **Mn**: mammilary nuclei, **SN**: substantia nigra, **Th**: thalamus, **VTA**: ventral tegmental area, **VThn**: ventral thalamic nuclei

Figure 2.7.5

A. Northern blot analysis of Tg TH HaPrP mice

RNA samples from dissected brain regions from Tg TH 15 (litter 2) and whole brain RNA samples from F1 and Tg NSE 20 (litter 5) mice, probed with the SV40 KpnI-XhoI SV40 fragment.

1. cerebellum Tg TH 15 (litter 2)
2. brain stem Tg TH 15 (litter 2)
3. coliculi Tg TH 15 (litter 2)
4. hippocampus Tg TH 15 (litter 2)
5. cortex and striatum Tg TH 15 (litter 2)
6. rest of brain Tg TH 15 (litter 2)
7. olfactory bulb Tg TH 15 (litter 2)
8. -
9. F1 brain
10. Tg NSE 20 brain (litter 5)

B. RT-PCR analysis of Tg TH HaPrP mice

PCR from Tg TH HaPrP genomic DNA sample was performed as a positive control revealing the expected 300 bp PCR product. Negative control PCR reactions were performed on genomic DNA from F1 and non-Tg littermates. RT-PCR was performed on dissected brain regions of Tg TH 15 (litter 4) brain. Reactions where the RT step, the or addition of RNA was ommited were also included in order to eliminate false positives due to DNA contamination in the RNA samples or contamination of the reagents.

1. 1 kb ladder
2. F1 genomic DNA
3. non-Tg littermate of Tg TH 15 (litter 4) genomic DNA
4. Tg TH 15 (litter 4) genomic DNA
5. cortex (no RT)
6. hippcampus (no RT)
7. cortex
8. hippocampus
9. rest of brain
10. olfactory bulb
11. rest of brain (no RT)
12. olfactory bulb (no RT)
13. no RNA

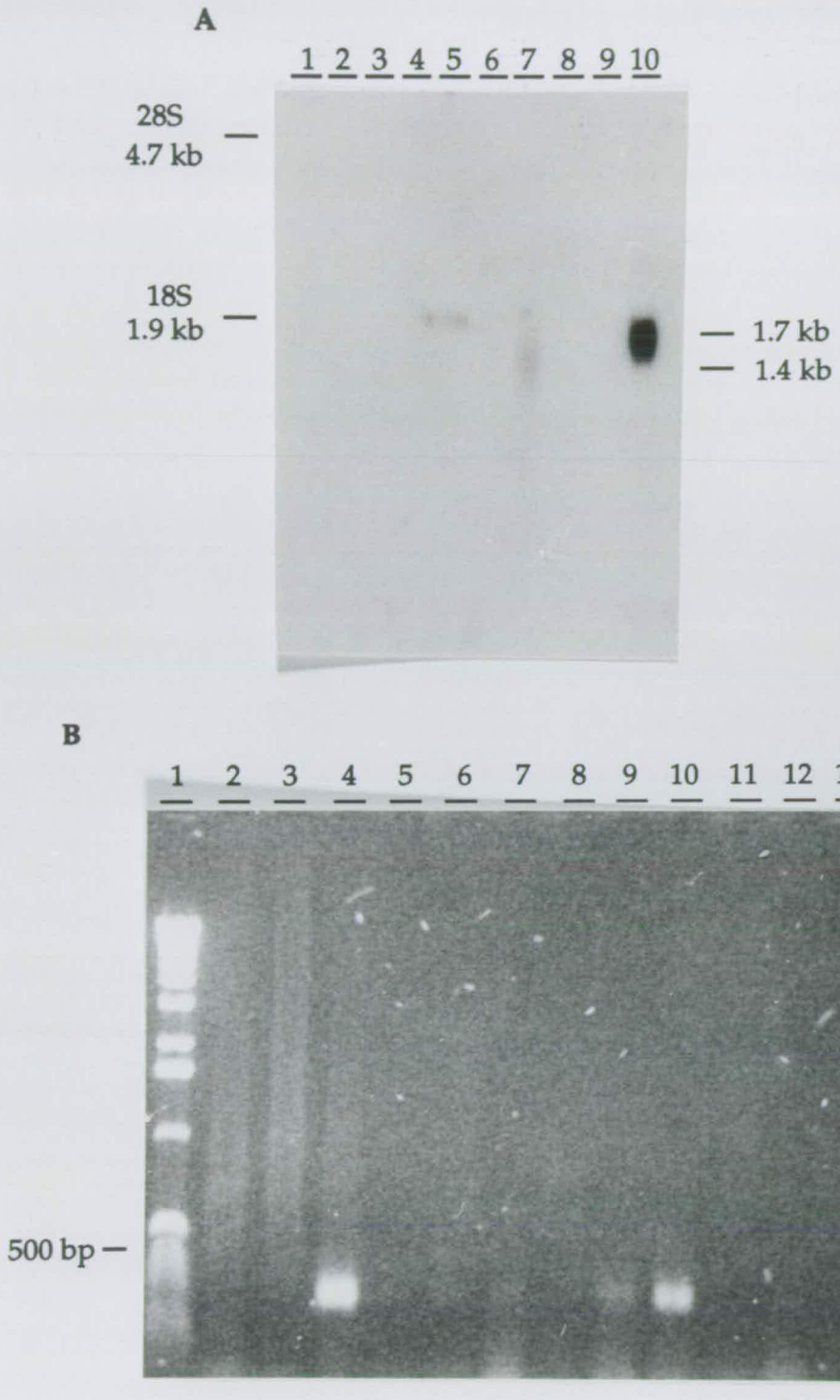

A
1 2 3 4 5 6 7 8 9 10
28S
4.7 kb
18S
1.9 kb
1.7 kb
1.4 kb
B
1 2 3 4 5 6 7 8 9 10 11 12 13
500 bp

figure 2.7.5

be due to cross-contamination between the brain areas during the dissection procedure. Nigrostriatal projections could also be present in different brain regions apart from the ventral tegmental area and the substantia nigra. Very faint bands were also sometimes detected in the reactions where the RT step was omitted, suggesting that there could be some residual DNA in the RNA samples. The primers used for the PCR step were two of the oligos used for the *in situ* hybridisation, Ha-s and SV40-a, in the HaPrP sequence and in the SV40 sequence respectively, giving a 300 bp PCR fragment.

The transgene-specific signal detected by *in situ* hybridisation was very low in the olfactory bulb and there were indications that the signal observed in other regions could be background hybridisation. Northern blot analysis also revealed very low hybridisation signal in the olfactory bulb. The results obtained by RT-PCR approach were inconclusive. Therefore, it was decided not to pursue further the study of Tg TH HaPrP lines.

2.8 Conclusions

Analysis of the Tg NSE HaPrP lines demonstrated only low levels of transgene PrP mRNA expression in comparison to endogenous MoPrP mRNA expression. No comparison was made with endogenous NSE mRNA expression. However, low transgene mRNA levels, in comparison to endogenous NSE mRNA, were obtained in a previous transgenic model where the same 1.8 kb fragment containing 5' flanking sequences of the NSE gene was used (ForssPetter *et al.*, 1990). The data presented in section 2.6 also suggest that if the mRNA produced by the transgene was translated as efficiently as the endogenous PrP message in the brain, the protein produced should be detectable by the method applied here in, at least, Tg NSE line 20 (the amount of transgene-produced mRNA is 15% in comparison to endogenous MoPrP). No HaPrP protein was detected in any of the brain samples tested from Tg NSE HaPrP lines. Similarly, in unpublished studies in which the HuPrP ORF was under the control of the 1.8 kb NSE promoter fragment mRNA expression but no protein was detected (Figures 2.6.1, 2.6.3 and Dr. P. Estibeiro and Dr. O. Windl personal communication). Also, results

obtained from transgenic studies where the sheep PrP ORF was used under the control of the Thy-1 promoter suggested that possibly the sheep PrP ORF was not sufficient for PrP protein expression (Dr J. Hope and Dr H. Baybutt, personal communication). It should be noted that the sheep PrP would be detected only if the signal produced was high enough to be distinguished over the signal produced by the endogenous MoPrP, since there was no sheep specific anti-PrP monoclonal antibody available.

Another attempt to generate transgenic mice with a construct that contained only the HaPrP ORF had failed, but the reasons for this were not clear (Scott *et al.*, 1989). A fragment containing putative PrP promoter sequences that had not been characterised was used to drive HaPrP expression; no RNA data were presented.

One interpretation of these results was that there could be a block at the level of translation due to the use of the isolated PrP ORF. It was hypothesised that the 5' and 3' untranslated sequences of PrP might contain important regulatory sequences.

Because the analysis of the TH HaPrP lines involves complicated techniques, and the analysis of the Tg NSE lines indicated that there could be a problem with the use of the PrP ORF fragment in the transgenic constructs, analysis was restricted to one of the three TH HaPrP transgenic lines generated. *In situ* hybridisation and Northern blot analysis indicated that there was probably extremely low PrP mRNA expression in at least some of the catecholaminergic neurons (olfactory bulb). Therefore, it was decided that the TH HaPrP lines would not be pursued and no more transgenic lines would be generated with either construct. Recent experiments (Min *et al.*, 1994) demonstrated that a 9 kb fragment of the 5' flanking sequence of the rat TH gene is necessary to drive high-level, specific expression of a reporter gene (lacZ) in transgenic mice.

Although the data presented here suggested that the open reading frame alone could be insufficient for PrP protein expression and other elements of the PrP gene (missing from constructs previously tested) could be necessary for adequate translation and translocation of the mRNA, several studies demonstrated that PrP ORF containing constructs appeared to produce PrP protein in cell culture, though sensitive detection methods were necessary (section 1.12.2). However, in

some cases PrP mRNA, but no PrP protein, was detected (Scott *et al.*, 1988). The role of the 5' and 3' untranslated sequences of PrP has not been addressed; all successful transgenic models were generated with cosmid-based constructs and constructs used in cell culture contained only the PrP ORF (section 1.12). PrP intron sequences could also be important for PrP protein expression. Apart from influencing transcription efficiency, intron sequences could be important for RNA routing. Also, splicing could contribute to the stability of PrP mRNA.

In retrospect, there are two trivial reasons that account for the failure to express PrP protein in the Tg NSE HaPrP transgenic mice:

a. The polylinker that lies between the NSE cap-site and the PrP reading frame contains an ATG start codon. Since this is out of frame relative to the PrP sequence, polypeptides initiated at this site will not react with anti-PrP sera.

b. The PrP cDNA sequence (Oesch *et al*, 1985) employed in the constructs lacks the first 32 nucleotides, including the initiator ATG codon and consequently any protein translated from it would lack the first 14 N-terminal aminoacids (initiation at the second ATG, 42 nucleotides downstream the first ATG). In effect the signal peptide would be reduced in length from 22 to 8 aminoacids. This could effect PrP protein levels through a variety of routes, such as translation efficiency of mRNA and altered stability of mRNA and/or protein, either intrinsic to the molecule or due to an altered cellular distribution. The same PrP fragment was used also in the TH HaPrP construct.

To investigate possible effects of the 5' and 3' UTR of HaPrP and the presence of an intron on translation efficiency, a series of constructs was tested in cultured cells. At the same time the effect of the N-terminus truncation of the HaPrP fragment on protein levels was assesed.

CHAPTER 3

The influence of the 3' and 5' untranslated regions of hamster PrP on the translation of lacZ

3.1 Eukaryotic mRNA regulation-control of translation

3.1.1 Mechanisms that influence initiation of translation

The regulation of mRNA translation is recognised to be one of the crucial steps for modulating gene expression. There are several features that can influence the initiation of translation, pre-mRNA processing and mRNA stability, subcellular localization and degradation.

In eukaryotes the initiation of translation is modulated by the phosphorylation-dephosphorylation of the translation initiation factors (Sonenberg, 1994; Merrick, 1992; Jackson, 1993) and five features of the mRNA: the 5' m^7G cap structure, the primary sequence (context) around the initiating AUG codon, the position of the AUG codon (whether it is the first AUG in the mRNA), the secondary structure upstream and downstream from the AUG codon and the length of the leader sequence (Kozak, 1991a; Kozak, 1991b).

The 5' cap structure is present in most eukaryotic mRNAs and there is strong evidence suggesting that ribosomes bind at, or near the cap and 'scan' the mRNA in a linear 5'-3' fashion, until they encounter an AUG initiator (in the right context, see below) (Kozak, 1991a; Kozak, 1991b). Cap-independent, internal initiation of translation can also occur. Uncapped mRNAs of picornaviruses and several vertebrate and invertebrate cellular mRNAs contain internal ribosomal entry sites (reviewed in Sonenberg, 1994, Kozak, 1991a, Jackson, 1993). It has been demonstrated that ribosomes can bypass a segment of the 5' UTR (mosaic virus mRNA) suggesting an alternative mechanism to the linear scanning model (Sonenberg, 1994). The cap structure is also important for mRNA stability and transport (reviewed in Sachs, 1993, Kozak, 1991b).

Comparison between eukaryotic mRNAs and mutagenesis of the nucleotides surrounding the initiator AUG codon revealed that the most common sequence for initiation of translation is GCC$^{A}/_{G}$CCAUGG ('strong', or favourable context) (Kozak, 1991a; Kozak, 1991b; Kozak, 1989). The most conserved nucleotide is a purine (usually A) at position -3 (3 nucleotides upstream of the AUG) and G at +4 (the A of the AUG being defined as position +1). The contribution of the other positions (-1, -2, -4, -5 ,-6) is more obvious when nucleotides at positions -3 and +4 are unfavourable (Kozak, 1989). In that case nucleotides even further upstream of the AUG (-7 to -9) can contribute to the efficiency of translation (GCCGCC$^{A}/_{G}$CCAUGG, optimal consensus), but those closer to the AUG have greater influence. Also, these nucleotides enhance translation only when they occur together and at a precise distance from the AUG (Kozak, 1987; Kozak, 1989). The translation of mRNAs that do not have a 'strong' consensus sequence is regulated and therefore usually they encode regulatory proteins (Kozak, 1991a) presumably because they are required only at low levels.

According to the scanning model the first AUG in the mRNA sequence is the initiator AUG, when it is in a 'strong' context. However, there are several cases (mainly in viral mRNAs) where two proteins are produced from the same mRNA. This occurs when the context of the first AUG is unfavourable, causing 'leaky' scanning and initiation from a second AUG. In vertebrates, promoter switching or alternative splicing is usually necessary to produce two mRNAs and (possibly) two proteins from the same gene (Kozak, 1991a; Kozak, 1991b; Geballe and Morris, 1994). Evidence from yeast suggest that the first AUG in the mRNA sequence is the initiator codon, even if translation is very weak due to unfavourable context (reviewed in Kozak, 1989). These data indicate that the existence of upstream AUG would inhibit translation from downstream AUG in eukayotic mRNAs (apart from viral mRNAs). However, when the ORF following the first AUG is very short the ribosomes can reinitiate translation at a downstream AUG (Kozak, 1991b; Kozak, 1987a; Geballe and Morris, 1994). The intercistronic length is important for the efficiency of the reinitiation by eukaryotic ribosomes (Kozak, 1987b; Geballe and Morris, 1994).

The secondary structure surrounding the initiator AUG is also crucial. Extensive secondary structure upstream of the AUG has an

inhibiting effect on translation efficiency. Impairment of translation depends on the distance between the structure and the cap (inhibition is stronger when the secondary structure is nearer to the cap) and the free energy of the structure. In contrast, secondary structure downstream of the AUG may assist recognition of the initiator codon, perhaps by slowing ribosome scanning, contributing to the fidelity of translation initiation. This may sometimes compensate for an unfavourable AUG context (Kozak, 1991a; Kozak, 1991b). Presence of secondary structure 5' to an AUG codon might enhance initiation from upstream AUG codons or even from cryptic non-AUG codons (Kozak, 1991a).

Apart from the structure of the 5' leader sequence, its length is also crucial. If the AUG is positioned too close to the cap (less than 20 (Kozak, 1991b) or 40 (Geballe and Morris, 1994) nucleotides) and there is no substantial secondary structure downstream of that AUG, bypassing is thought to be favoured, increasing initiation from a downstream AUG. Further lengthening of the leader sequence has a dramatic increase on translation efficiency. Generally, long, unstructured leader sequences (low G content) preceding the AUG codon appear to be necessary for high levels of translation (Kozak, 1991a; Kozak, 1991b).

3.1.2 The influence of 3' and 5' untranslated regions (UTR) on mRNA regulation and control of translation.

Translation of the mRNA can be regulated by sequences in the 5' and 3' untranslated regions (UTRs) involving protein-mRNA interactions. In eukaryotes when the mRNA is regulated by 5' UTR motifs, the most important aspect is the distance from the cap (reviewed in Jackson, 1993). The regulation of ferritin mRNA by iron has served as a model for studying the inhibition of translation by specific structures in the 5' UTR (reviewed in Sonenberg, 1994; Jackson, 1993). Regulation depends on a stem-loop structure, the iron responsive element (IRE) which lies within 40 nucleotides of the cap structure. A protein termed iron regulatory factor (IRF), or IRE-binding protein (IRE-BP) binds to the IRE when iron levels are low and inhibits ferritin mRNA translation. There is evidence suggesting that changes in availability of iron involve changes in the iron-sulfur cluster in the IRF, which alter the RNA-

binding activity of the protein (Haile *et al* 1992). It has also been demonstrated that nitric oxide inhibits ferritin translation by increasing binding of IRF to the IRE either through chelation of iron, or by affecting the iron-sulfur cluster (Sonenberg, 1994). The IRE has to be close to the cap, whereas the distance between the IRE and the initiator AUG is not significant (Stripecke *et al* 1994; Jackson, 1993). Another IRE has been identified in the 5' UTR of the mRNA of erythroid 5-aminolevulinic acid-synthase (rate limiting enzyme of heme biosynthesis) (Dandekar *et al* 1991), indicating that iron might regulate several mRNAs that are involved in iron metabolism pathways.

Autoregulation of translation by repressor protein binding to the 5' UTR has been reported in the case of thymidylate synthase, which binds to its own mRNA downstream of the cap at a sequence that includes the initiator AUG (reviewed in Sonenberg, 1994; Stripecke *et al* 1994). Dihydrofolate reductase also binds at the 5' of its own mRNA, inhibiting translation (cited in Sonenberg, 1994).

In vitro studies have indicated that inhibition of translation of a family of seven related Drosophila mRNAs during spermatogenesis depends on repressor protein binding to a 12 nucleotide motif located 28 nucleotides downstream of the cap (reviewed in Jackson, 1993).

Several examples of the involvement of 3' UTR sequences in the regulation of translation have been reported.

A 60 nt motif has been found in the extreme 3' of protamine mRNA in mice which is thought to be necessary for temporal regulation (inhibition) of translation in the developing spermatides. The proteins that bind to this motif are homologous to RNA-binding proteins found in Xenopous oocytes and are found only in cells where protamines are not translated (reviewed in Jackson, 1993).

The synthesis of the sex determination protein of C. elegans tra-2 is negatively regulated by a 28 nucleotide direct repeat element in the 3' UTR of the mRNA (reviewed in Sonenberg, 1994).

Synthesis of rabbit erythroid 15-lipoxygenase is negatively regulated by the binding of a 48 kD protein to a region of 10 tandem repeats of similar pyrimidine-rich 19 nucleotide motifs in the 3' UTR (Ostareck-Lederer *et al* 1994).

It has been demonstrated that part of the 3' UTR of α-tropomyosin mRNA suppresses tumor formation (Rastinejad *et al* 1993) and also that

the 3' UTRs of troponin I, tropomyosin and α-cardiac actin mRNAs are trans-acting regulators that inhibit cell division and promote differentiation (Rastinejad and Blau, 1993).

The utilisation of two alternative polyadenylation sites in the 3' UTR of the amyloid precursor protein (APP) mRNA provides another example of control of translation by 3' UTR sequences (De Sauvage *et al.*, 1992). The longer mRNA that uses the second polyadenylation site was shown to be translated more efficiently *in vitro* than the shorter one. Furthermore, the sequence between the two sites increased translation of reporter genes (chicken lysozyme and chloramphenicol acetyltransferase) when cloned downstream of their coding regions to produce chimeric mRNAs.

Sequences in the 3' UTR have also been shown to influence mRNA stability and degradation. AU-rich motifs (consensus AUUUA), in the 3' UTRs of granulocyte-macrophage colony stimulating factor, lymphokine and protooncogene mRNAs are thought to make them unstable (reviewed in Jackson, 1993, Sachs, 1993). c-myc and c-fos mRNAs have destabilising elements in the ORF and destabilising AU-rich elements in the 3' UTR, responsible for the high turnover of their mRNAs. Stimulation of degradation by AU-rich elements requires translation of the mRNA (reviewed in Sachs, 1993). Binding of two proteins to AU-rich elements in the 3' UTR of c-myc has been shown to promote degradation (cited in Jackson, 1993).

In the 3' UTR of the transferrin receptor mRNA there are five secondary structure elements resembling the IRE in the 5' UTR of ferritin mRNA. Binding of the IRF protein to these structures under conditions of iron starvation appears to stabilise the mRNA (Koeller *et al* 1989).

Sequences in the 3' UTR can also be responsible for correct localization of mRNAs in the cell. Examples are the oskar and bicoid mRNAs in Drosophila eggs that need to be localised to the posterior and the anterior end respectively. Transport and retention in the final location are achieved through RNA-protein interactions that involve binding of imperfect, but extended double-stranded mRNA sequences to the RNA-binding protein, staufen (reviewed in Jackson, 1993). Specific localization of α-actin and ß-actin mRNAs in the differentiating myotubes requires a 63 nucleotide region (including a 12 nucleotide

motif) in the 5' end of the 3' UTR of the mRNAs that probably interacts with an as yet unidentified RNA-binding protein (reviewed in Jackson, 1993).

Other important signals present in the 3' UTR are motifs known as cytoplasmic polyadenylation elements (CPEs) in Xenopus, or adenylation control elements (ACE) in the mouse, which control shortening of the poly(A) tail when the mRNA is masked at the first stages of oogenesis and the lengthening of the poly(A) during maturation, when the mRNA is translated (reviewed in Sachs, 1993). All mRNAs contain a polyadenylation addition signal, AAUAAA in the 3' UTR. Evidence mainly based on studies in yeast demonstrate that deadenylation might be a prerequisite for mRNA degradation (reviewed in Kozak, 1987b). Apart from contributing to the mRNA stability, the poly(A) tail appears to enhance translation through interactions with the 5' cap structure (reviewed in Sonenberg, 1994). The poly(A) tail increases translation of capped mRNAs only and the 5' cap promotes translation of poly(A)+ mRNAs much better than poly(A)- mRNAs. Other studies suggest that interactions between sequences in the 3' UTR and sequences in the 5' UTR stimulate translation. Whether this involves direct interaction between these sequences or interaction with another protein, which could be a component of the translation initiation machinery, is not clear. A poly(A)-binding protein (PAB), that could mediate this interaction has been characterised in yeast. Interaction between the 5' and 3' UTR is also supported by electron microscopy studies (reviewed in Sonenberg, 1994).

3.2 The 5' and 3' UTR of PrP mRNA

The importance of 5' and 3' UTR sequences on mRNA regulation and translation was discussed in section 3.1. Data obtained using the transgenic mice generated with constructs that did not contain 5' or 3' UTR sequences of HaPrP, or intron sequences, indicated that these sequences were possibly involved in controlling PrP translation (section 2.8). Investigating their role could possibly also give some insight into the function of PrP, since there is evidence suggesting that tightly regulated genes are usually regulatory proteins such as cell surface

receptors, growth factors, transcription factors and cytokines, or protooncogenes, or proteins involved in development and differentiation (Sonenberg, 1994; Kozak, 1991a; Geballe and Morris, 1994).

A remarkable feature of the PrP genes is that the 3' UTR sequence is conserved between mammals. Comparison of the 3' UTR of PrP mRNA of sheep with that of man and rodent (hamster and mouse) revealed certain regions that were up to 90% homologous (Goldmann *et al.*, 1990, personal observations). Figure 3.2.1 shows the approximate regions of homology. The length of the most conserved regions was estimated through sequence alignments done with 'pileup' GCG7, University of Wisconsin Genetics Computer Group software package. The approximate length for each region was: A=180 bp, B=320 bp, C=240 bp, D=520 bp, E=440 bp, F=1440 bp G=160 bp. There are two sheep specific insertions (D and F regions, Figure 3.2.1), region B is not present in rodent PrP and region C is not present in human PrP. Because these comparisons revealed high conservation between certain regions, secondary structure analysis was performed in order to identify possible similarities in the structure of these regions ('fold' and 'squiggles' programs, of the software package mentioned above, were used). No conserved motif was found in any of the regions that showed homology on the primary sequence level. Also there was no similarity to known regulatory motifs (section 3.1). Primary sequence comparison between more PrP sequences in addition to those previously studied, including bovine, mink and rat PrP, revealed that only shorter regions within the regions previously defined exhibited over 50% homology. For example, in the 5' end of region B there is a 55 nucleotide stretch (region B1) that shows 60% homology between the seven PrP sequences. Again, secondary structure analysis of these highly conserved regions failed to reveal a conserved secondary structure motif, although some structures exhibited similarity. Furthermore, the secondary structure analysis data showed that the same sequence would appear to have a different structure if different parts of the surrounding sequences were included in the analysis. Therefore, no conclusions could be drawn. A more informative approach would be to test whether the 3' UTR (and subsequently specific regions of the 3' UTR) are important for mRNA regulation and translation and then try to identify secondary structure motifs in these sequences.

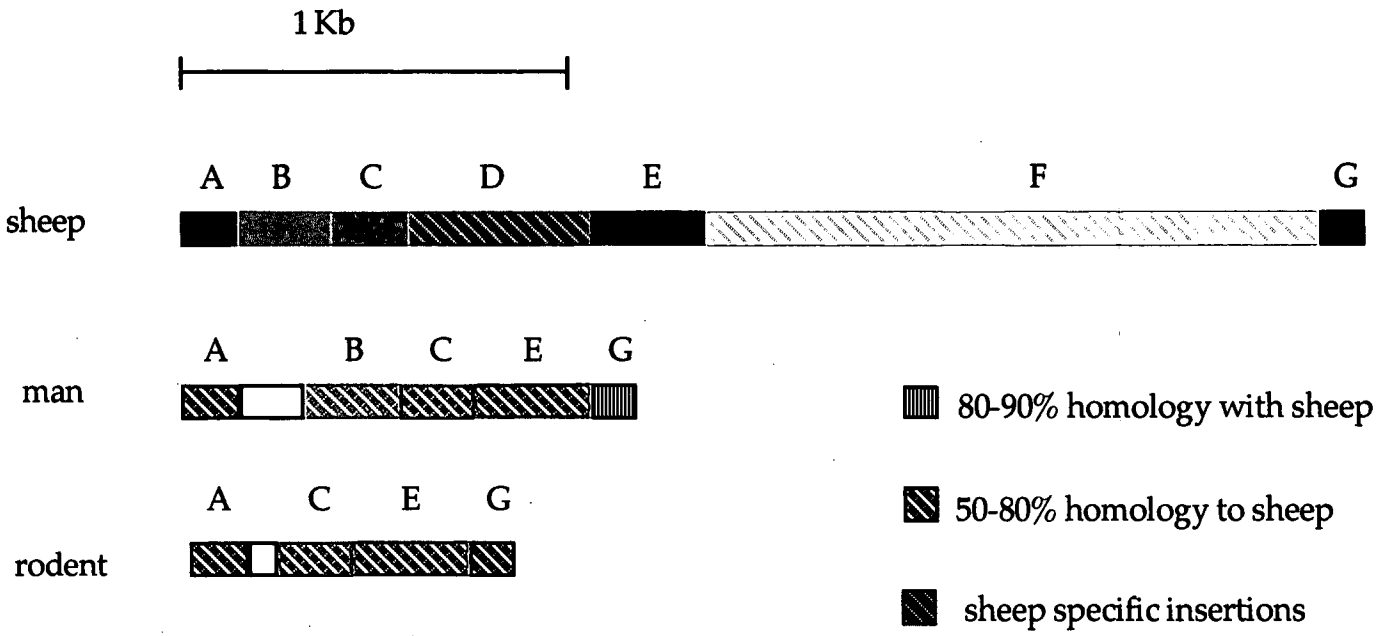


Figure 3.2.1 Comparison of 3' UTR PrP mRNA sequences of man and rodent with the sheep. The different hatching represents different percentage of homology with the sheep sequence. This figure is based on (Goldmann *et al* 1990, Figure 6).

The 5' UTR of PrP is not well defined for most species and primary sequence comparison between known 5' UTR sequences revealed less than 50% homology. The existence of two upstream exons (i.e. in mouse PrP) contributes to the diversity of the 5' UTR PrP sequences. These features did not assist predictions as to whether any of these sequences could be involved in regulating translation by secondary structure inhibition or by regulatory proteins binding to certain motifs. The best approach would be to test the effect of different parts of the 5' UTR on translation efficiency.

In some previous experiments, the initiation of translation and leader sequence of HaPrP were replaced with the 'optimal' consensus sequence (see Figure 3.5.2) and the human cytomegalovirus 5' leader sequence from pEE6hCMV vector (appendix 2), in order to obtain high level PrP protein production (Dr L. Wightman and Dr A. Bennett, personal communication). The HaPrP fragment (consisting of the ORF only) with the altered initiation of translation sequence, had been cloned in the polylinker of pEE6hCMV as a HindIII-EcoRI fragment. Once this construct became available, it was used in cell culture in parallel with other constructs containing different parts of 5' UTR of HaPrP (see sections 3.5.1 and 4.2 and 4.5).

3.3 Investigation of the role of the 5' and 3' UTR of HaPrP on translation

To resolve the issues concerning PrP translation and possible presence of regulatory elements in the 5' and 3' UTR of PrP, the effect of these sequences on the translation of a reporter gene (lacZ) and on HaPrP was determined. lacZ would provide an easy and accurate assay system, but any regulation due to specific interactions between PrP sequences would be revealed only when 3' and 5' UTR PrP sequences are used together with the PrP ORF.

Sequences investigated were the 3' UTR of HaPrP, the 5' UTR of HaPrP in exon 2 and the complete HaPrP 5' UTR (exon 1 and small part of exon 2). Also, the effect of changing the initiation of translation consensus sequence and changing the leader sequence together with the

addition of a generic intron was investigated. Intron sequences are known to regulate transcription and their presence has been shown to be important both *in vivo* and *in vitro* (section 2.1). Although there is no clear evidence demonstrating that introns can positively regulate translation of mRNA (though when introns are not removed by splicing they can inhibit translation (Kozak, 1991a)), it would be interesting to investigate whether the presence of an intron can influence PrP translation.

The effect of of these sequences on the translation of lacZ is presented in this chapter and the effect on the translation of HaPrP is presented in chapter 4.

3.4 The effect of the HaPrP 3'UTR on lacZ translation efficiency

3.4.1 Generation of pCEPlacZ and pCEPlacZ3'UTR constructs

In order to identify possible translational control elements in the 3' UTR of HaPrP, the effect of the addition of the 1.2 kb 3' UTR of the HaPrP at the 3' end of the coding region of lacZ was investigated.

The expression vector used for cloning of the different fragments was pCEP4 (Invitrogen, appendix 2), which has the human cytomegalovirus (hCMV) promoter and an SV40 poly(A) sequence at either side of the polylinker. The lacZ gene was derived from pSV-ß-galactosidase (pSV-ß-gal) vector (Promega, see appendix 2). The 3287 bp HindII-EcoRI lacZ fragment was excised from pSV-ß-galactosidase vector and cloned into the polylinker of pSP72.poly1 vector (appendix 2). pSP72.poly1 was used as an intermediate vector for the cloning of lacZ because pCEP4 does not have an EcoRI site in the polylinker. pBluescriptII KS- could not be used because it already contains a portion of the lacZ gene, and cloning the lacZ gene could cause recombination between homologous parts and instability. The 320 bp lacZ fragment was PCR amplified with primers containing appropriate sites. The forward primer lacZ.1 (appendix 1) included the EcoRI site (present in the lacZ gene) and lacZ sequence beyond this site and the reverse primer, lacZ.2 (appendix 1) included the sequence at the end of the lacZ gene, and introduced a NotI site. This fragment was cloned in the EcoRI-NotI sites

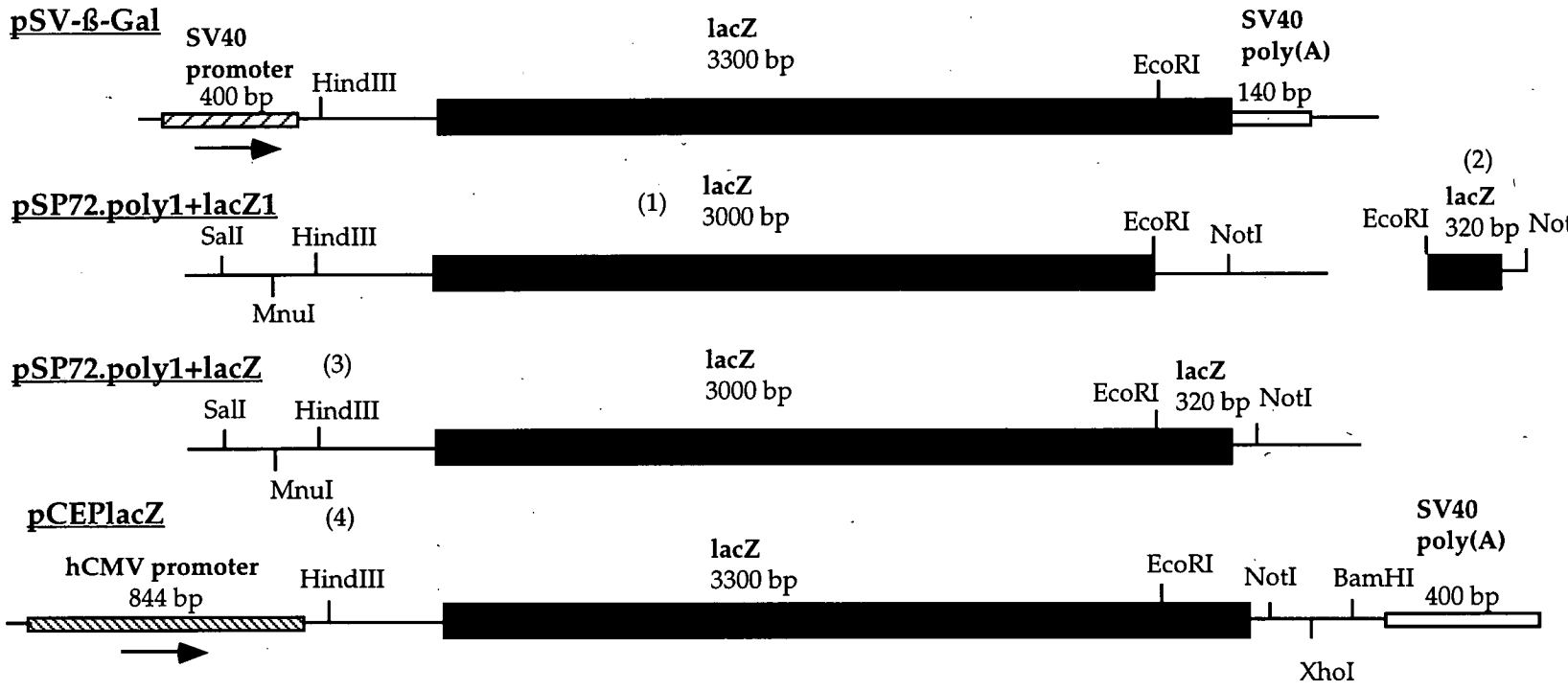


Figure 3.4.1 Generation of pCEPlacZ construct

(1) The HindIII-EcoRI fragment containing lacZ sequences was sucloned into pSP72.poly1, to generate pSP72.poly1+lacZ1

(2) PCR with appropriate primers gave the remaining 320 bp of lacZ, which was subcloned (3) into pSP72.poly1+lacZ1 as a EcoRI-NotI fragment to give pSP72.poly1+lacZ.

(4) The entire lacZ fragment was subcloned into pCEP4 as a HindIII-NotI fragment to generate pCEPlacZ.b.

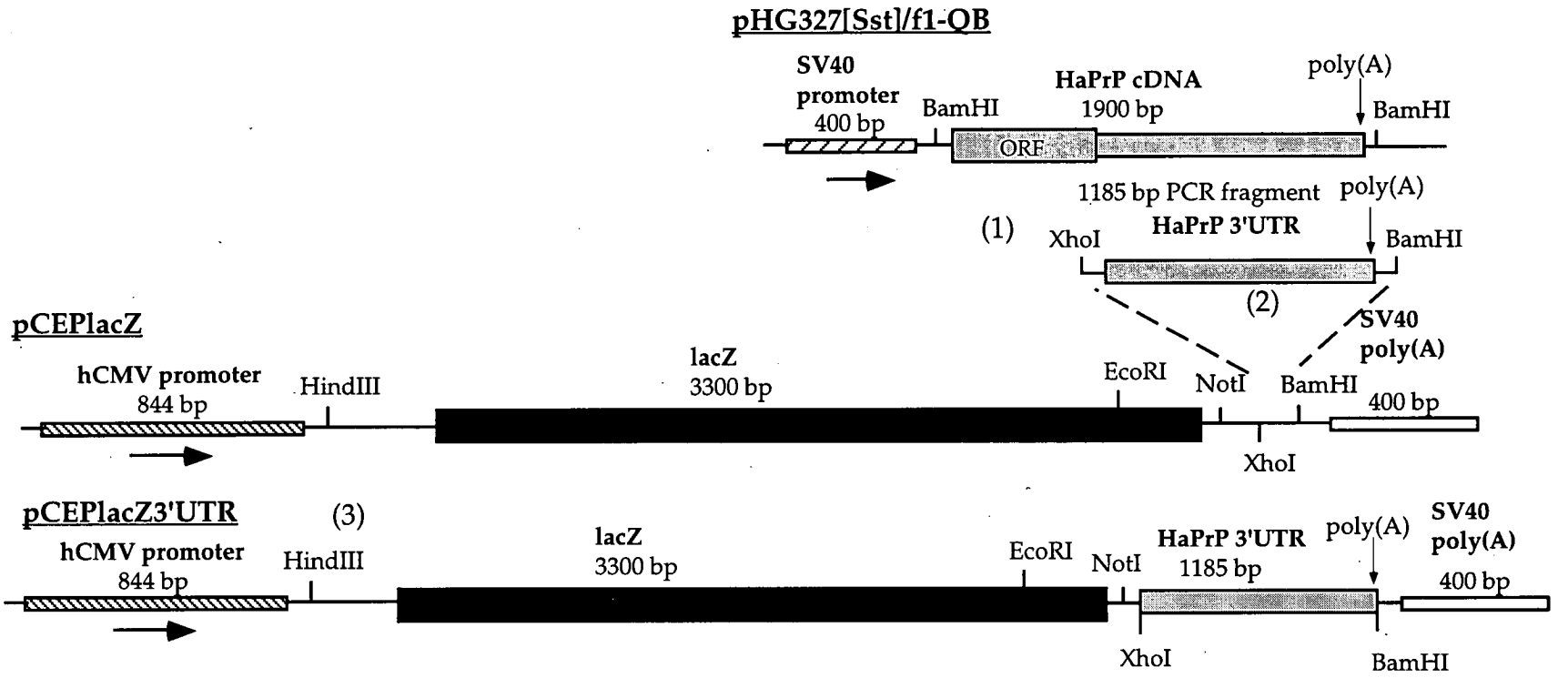


figure 3.4.2 Generation of pCEPlacZ3'UTR construct

(1) PCR with appropriate primers (introducing an XhoI and a BamHI site, see text) of the 1185 bp HaPrP 3' UTR fragment.

(2) Subcloning of the 1185 bp fragment into pCEPlacZ construct to generate the pCEPlacZ3'UTR construct (3).

of the polylinker of pSP72.poly1 where the 3287 bp lacZ fragment had already been cloned (Figure 3.4.1). The 320 bp fragment and the regions around the cloning sites were fully sequenced to confirm that no mutations had been introduced by PCR. The whole HindIII-NotI fragment was cloned into the polylinker of pCEP4 to generate the construct pCEPlacZ (Figure 3.4.1).

The 3' UTR of PrP was PCR amplified from pHG327[Sst]/fi-QB vector (see also section 2.3.1, appendix 2). The forward primer, 3'UTR.1 (appendix 1) corresponded to HaPrP sequences immediately beyond the HaPrP TGA stop codon and included a XhoI site and the reverse primer, 3'UTR.2 (appendix 1) was located beyond the poly(A) addition signal, 1185 nucleotides downstream the stop codon and included a BamHI site (Figure 3.4.2). pCEPlacZ3'UTR was generated by cloning the XhoI-BamHI HaPrP 3' UTR fragment downstream of the lacZ in pCEPlacZ (Figure 3.4.2).

3.4.2 Expression of lacZ-HaPrP 3' UTR fusion constructs

The effect of the HaPrP 3' UTR on the translation of lacZ was determined by measuring ß-gal enzyme activity in protein extracts from cos7 cells (monkey kidney cells) transfected with the lacZ constructs. Cells were transfected by calcium phosphate method (section 7.29.5) with pSV-ß-gal in parallel with the two constructs described above (section 3.4.1). Transfection with pCEP4 vector, (not containing lacZ sequences), as well as co-transfection of each construct with pCAT-control vector (Promega), containing the chloramphenicol acetyltransferase reporter gene, were always performed. RNA was extracted from one portion of the harvested cells and protein from the remainder. Northern blot analysis was performed with RNA from the transfected cell lines and the result was quantified by phosphorimager (chapter 7, introductory paragraph). The probe used was the HindIII-EcoRI fragment of lacZ (Figure 3.4.1). The transcript produced from pSV-ß-gal and pCEPlacZ construct is 3.7 kb and from pCEPlacZ3'UTR 4.9 kb. CAT and ß-gal activity assays were performed on the protein extracts of harvested cells (sections 7.27, 7.28). CAT activity assay served as a

Tables 3.4.1 and 3.4.2 Translation efficiency of lacZ-HaPrP 3' UTR constructs in transiently transfected cos7 cells
(Co-transfections with pCAT construct)

Table 3.4.1
The ratio of ß-Gal activity to lacZ mRNA indicates translation efficiency.

	relative to pSV-ß-gal			
	CAT activity	lacZ mRNA	ß-Gal activity	translation efficiency
construct		**a**	**b**	**b/a**
pCEPlacZ	0.68	0.25	0.29	1.16
pCEP lacZ3'UTR	0.95	0.63	0.55	0.87
pSV-ß-Gal	1	1	1	1.00
pCEP(control)	0.89	0	~0	

Table 3.4.2
Data from three experiments.

	translation efficiency relative to pSV-ß-gal			
construct	**1**	**2**	**3**	**average**
pCEPlacZ	1.16	0.95	1.21	1.1
pCEP lacZ3'UTR	0.87	0.91	1.15	0.9
pSV-ß-Gal	1	1	1	1

control for transfection efficiency levels. Measurements of ß-gal mRNA levels, CAT and ß-gal enzyme activity were first expressed in relation to the values obtained in parallel by co-transfection with pSV-ß-gal and pCAT. The ratio of ß-gal activity to lacZ mRNA would give the translation efficiency for each construct.

One experiment is presented in detail as an example (Table 3.4.1, Figure 3.4.3) and the average of three experiments is presented in Table 3.4.2). The ß-gal mRNA levels presented in table 3.4.1 were estimated on the Northern blot shown in Figure 3.4.3.

The results obtained demonstrated that pCEPlacZ and pCEPlacZ3'UTR had a small insignificant difference in translation efficiency.

3.4.3 Conclusions

The results presented here indicate that the HaPrP 3' UTR does not have a significant effect on the translation of lacZ in cos7 cells, suggesting that it is not likely to contain any elements necessary for translation regulation. However, the possibility that the HaPrP 3' UTR has an effect only when combined with the HaPrP ORF, or an interaction between the 3' HaPrP UTR and the 5' HaPrP UTR cannot be excluded. Constructs containing different parts of the HaPrP gene would give an insight to this issue. Results considering constructs containing different HaPrP sequences are presented in chapter 4.

The effects of different 5' UTR portions of the HaPrP gene on the translation of lacZ were assessed by generating a series of 5' UTR HaPrP-lacZ fusion constructs and testing them in cell culture as presented in section 3.5.

Figure 3.4.3 Northern blot analysis of transiently transfected cos7 cells
Probed with the lacZ HindIII-EcoRI fragment.
Co-transfections with pCAT construct.

	construct
1.	pCEP
2.	pCEPlacZ
3.	pCEPlacZ3'UTR
4.	pSV-ß-gal

Figure 3.5.4 Northern blot analysis of transiently transfected cos7 cells
Probed with lacZ HindIII-EcoRI fragment.
Co-transfections with pCAT construct.

	construct
1.	pCEP
2.	pE2HaPßgeo
3.	pE1,2HaPßgeo
4.	pKoHaPßgeo

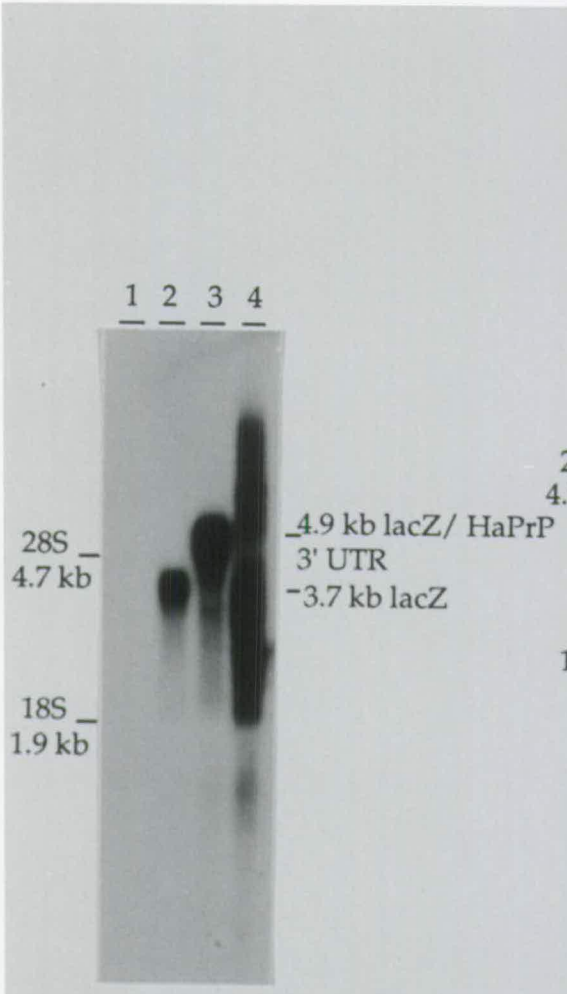


figure 3.4.3

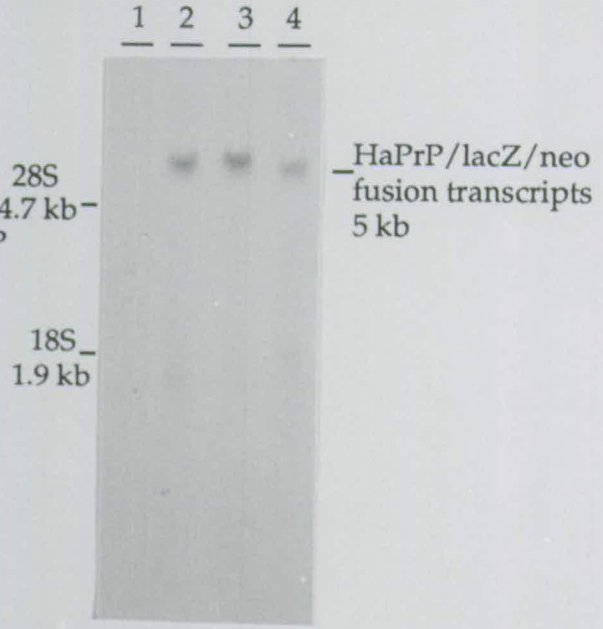


figure 3.5.4

3.5 Comparison of translation efficiency between 5' UTR HaPrP-lacZ fusion constructs

3.5.1 Generation of 5' UTR HaPrP-lacZ fusion constructs

Three different HaPrP 5' UTR-lacZ constructs were generated. Each contained a different 5' UTR sequence and the entire HaPrP ORF region fused to the lacZ gene. The stop codon of HaPrP and the first codon of lacZ were deleted (Figure 3.5.1), but the reading frame was preserved so as to create a functional HaPrP-lacZ fusion protein. This was possible by using PCR with appropriate primers for the HaPrP fragments prior to cloning in pßgeo vector (appendix 2). The sequence of pßgeo around the NruI cloning site at the beginning of the lacZ gene is shown in Figure 3.5.1. The three different 5' UTR sequences used were:

a. the 5' UTR sequence of HaPrP in exon 2 (10 nucleotides).

b. the complete 5' UTR HaPrP sequence including exon 1 (60 nucleotides) and the 5' UTR of exon 2 (10 nucleotides).

c. the 'optimal' initiation of translation consensus sequence directly upstream of the initiation codon of HaPrP.

The 'optimal' consensus sequence has been used previously in conjunction with the HaPrP ORF and has been demonstrated to produce high HaPrP expression in cell culture (Dr A. Bennett and Dr L. Wightman, personal communication). The sequence of the three different 5' regions are presented in Figure 3.5.2.

Genomic DNA from hamster brain was used to amplify by PCR a portion of HaPrP exon 2, containing the 5' UTR and the HaPrP ORF. RT-PCR on hamster brain RNA was applied in order to obtain the complete HaPrP 5'UTR (consisting of exon 1 and portion of exon 2) and the HaPrP ORF. The plasmid pEE6hCMVneoHaPrP (appendix 2) was used to amplify by PCR the HaPrP fragment with the 'optimal' consensus sequence. The reverse primer used for all three HaPrP fragments was 3'HalacZ (appendix 1, Figure 3.5.1) and included lacZ sequences up to the NruI site (including the NruI site and two more nucleotides, Figure 3.5.1). The forward primer for exon 2 was 5'ex2.a (appendix 1), for exon 1 was 5'ex1.a (appendix 1) and for the 5' 'optimal' consensus, 5'Ko (appendix 1).

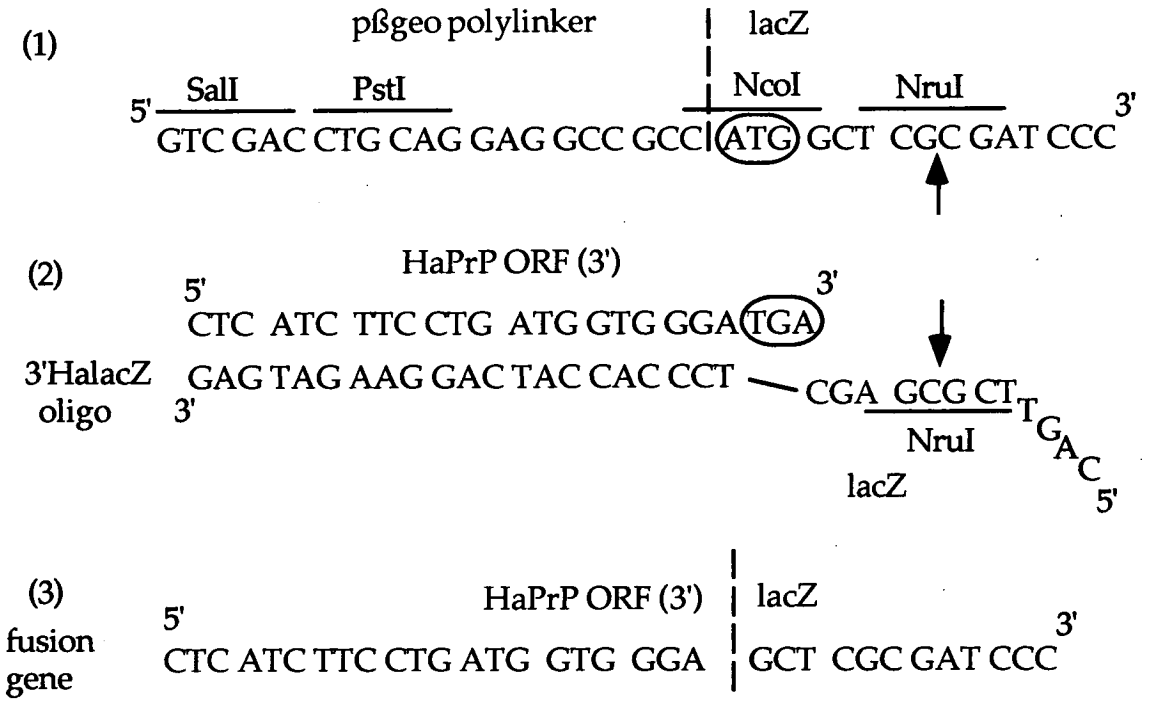


Figure 3.5.1 Fusion of 3' of HaPrP ORF with the lacZ (cloned in pßgeo vector)
(1) The polylinker of pßgeo . The SalI and the NruI sites were used for cloning the HaPrP PCR fragments.
(2) The 3' end of HaPrP ORF was fused in frame to the lacZ gene. This was achieved by using as a reverse primer to PCR amplify the HaPrP fragment, 3'HalacZ oligo, which included HaPrP and lacZ sequences (containing the NruI site).
(3) The stop codon of PrP and the initiation codon of lacZ have been deleted to generate an in frame HaPrP-lacZ fusion.

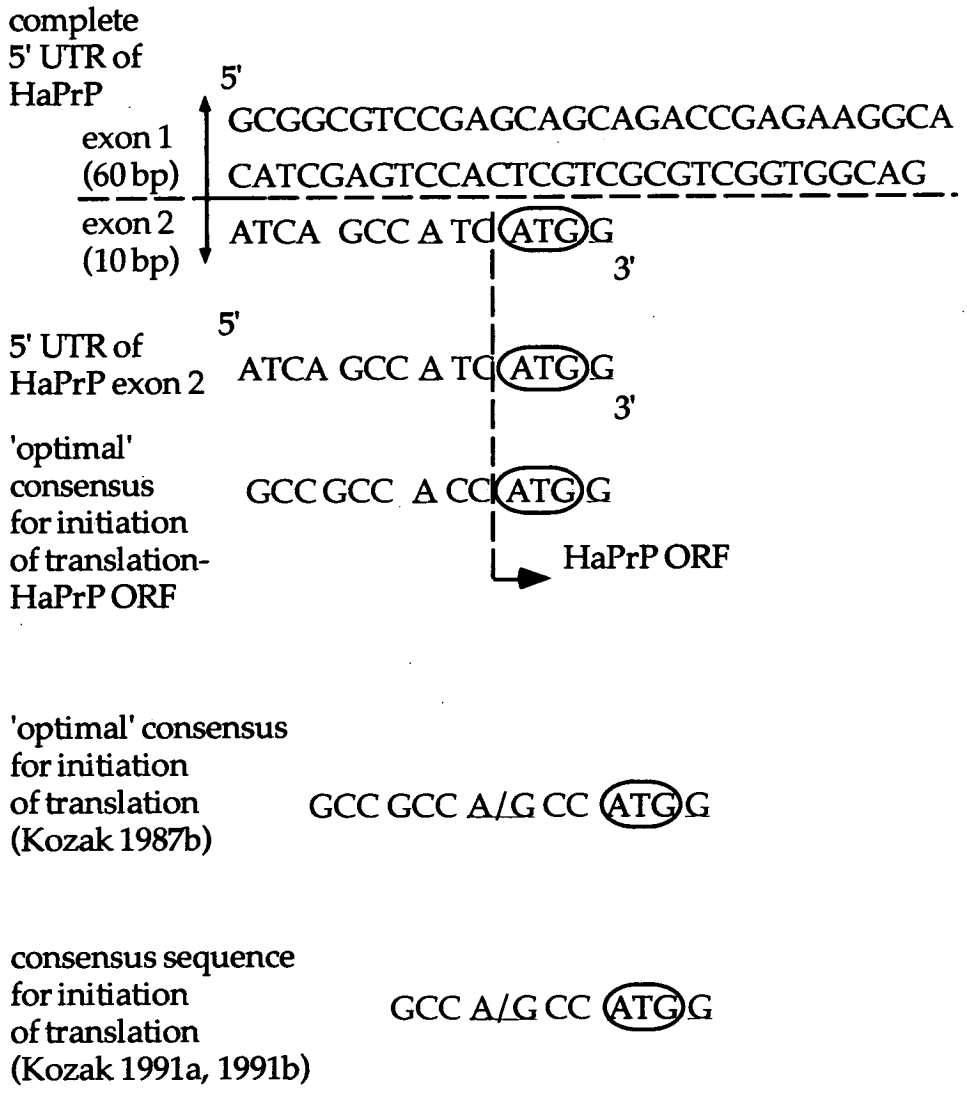


Figure 3.5.2 The three different 5' UTR sequences of the HaPrP-lacZ fusion constructs.

Three different 5' UTR sequences were used upstream of the HaPrP ORF. These 5' UTR-HaPrP ORF PCR fragments were fused to the lacZ gene in pßgeo (see Figure 3.5.1) to generate the three lacZ-HaPrP ORF fusion constructs presented in Figure 3.5.3.

The sequence surrounding the initiator AUG in each of the constructs is presented here in comparison to the consensus sequence (Kozak 1987b, Kozak 1991a, 1991b).

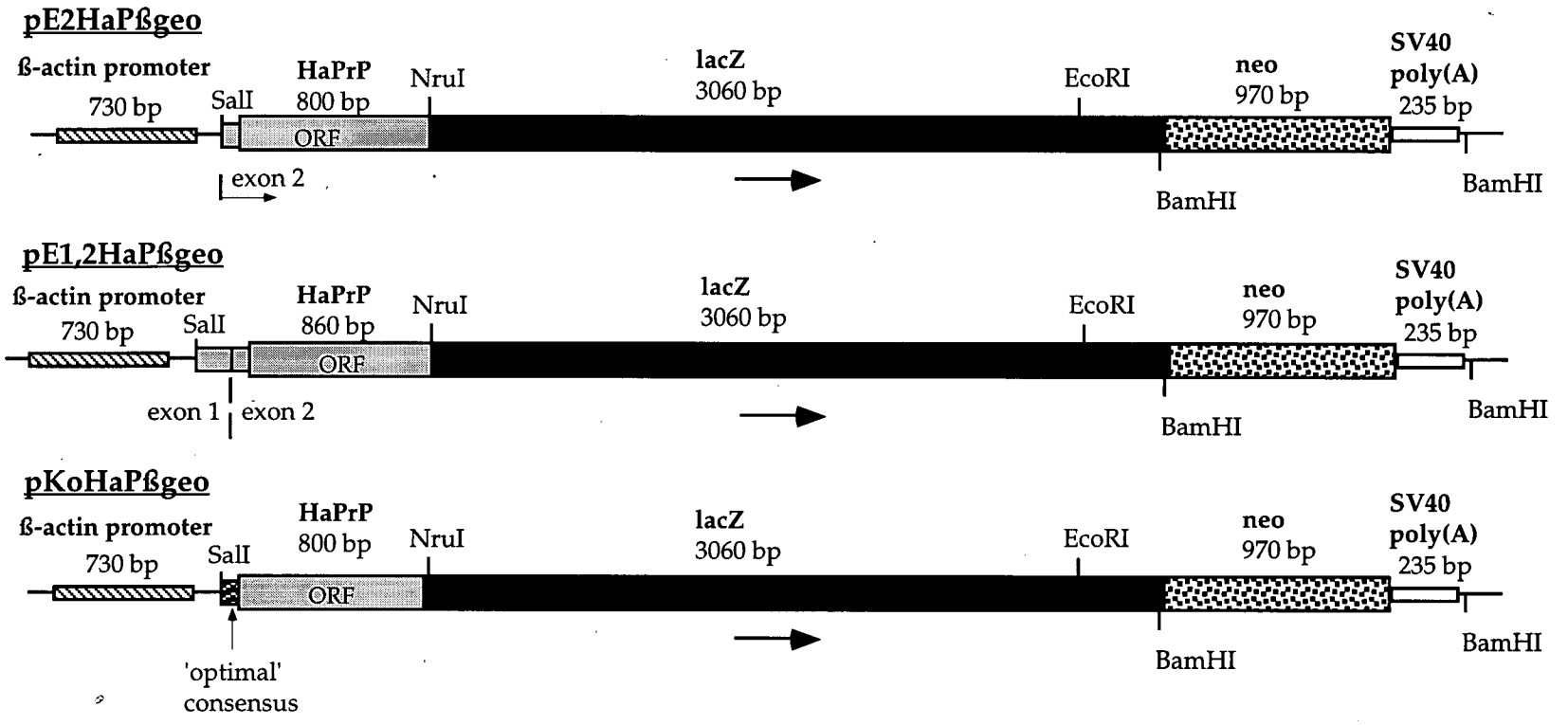


Figure 3.5.3 Generation of 5' UTR HaPrP ORF-lacZ fusion constructs

The 5' UTR of pE2HaPßgeo consists of exon 2 sequences. pE1,2HaPßgeo includes exon 1 (complete 5' UTR of HaPrP). In pKoHaPßgeo construct, the 5' UTR of HaPrP has been replaced with the 'optimal' consesnsus sequence (see text and Figure 3.5.2).

The three forward primers included a SalI site. After the PCR step, each of the three HaPrP fragments was cloned as a SalI-NruI fragment in pßgeo vector to generate the constructs pE2HaPßgeo, pE1,2HaPßgeo and pKoHaPßgeo as shown in Figure 3.5.3. The HaPrP fragment in each construct was fully sequenced, using internal HaPrP oligos, to ensure that no mutations had been introduced by the PCR step (the proofreading DNA polymerase UlTma (Perkin Elmer) was used) and the junction between HaPrP and lacZ was sequenced to confirm that the fusion was in frame.

3.5.2 Expression of 5' UTR HaPrP-lacZ fusion constructs

The two constructs containing different 5' ends of the HaPrP gene, pE2HaPßgeo (including the 5' UTR of exon 2) and pE1,2Haßgeo (including the 5' UTR of exon 1 and exon 2) and the construct made with the 'optimal' consensus sequence, pKoHaßgeo, were tested for translation efficiency after transfection in cos7 cells. As before the cells were co-transfected with the pCAT vector. The dark blue colour of transfected cells after X-Gal staining indicated that the lacZ gene was functional in all three constructs. Measurements of lacZ mRNA levels, CAT and ß-gal enzyme activities are shown relatively to the values obtained by co-transfection with pE2HaPßgeo and pCAT constructs (Table 3.5.1). The amounts of RNA presented in Table 3.5.1 were estimated by phosphorimager analysis based on the Northern presented in Figure 3.5.4. The Northern was probed with the HindIII-EcoRI lacZ fragment (Figure 3.4.1) and the transcript size is similar for the three constructs (5 kb). The ratio of ß-gal activity to ß-gal mRNA levels represents the translation efficiency for each construct. An example of the calculations is shown in Table 3.5.1 and the average of three experiments is shown in Table 3.5.2.

Tables 3.5.1 and 3.5.2 Translation efficiency of HaPrP 5' UTR-lacZ fusion constructs transiently transfected in cos7 cells
(Co-transfections with pCAT construct)

Table 3.5.1
The ratio of ß-Gal activity to lacZ mRNA indicates translation efficiency.

	relative to pE2HaPßgeo			
	CAT activity	lacZ mRNA	ß-Gal activity	translation efficiency
construct		**a**	**b**	**b/a**
pE2HaPßgeo	1	1	1	1.00
pE1,2HaPßgeo	1.25	1.12	1.95	1.74
pKoHaPßgeo	0.69	0.74	1.16	1.57
pCEP (control)	0.8	0	~0	

Table 3.5.2
Data from three experiments.

	translation efficiency relative to pE2HaPßgeo			
construct	**1**	**2**	**3**	**average**
pE2HaPßgeo	1	1	1	1
pE1,2HaPßgeo	1.74	1.68	1.97	1.79
pKoHaPßgeo	1.57	1.62	1.73	1.64

3.5.3 Conclusions

The HaPrP-lacZ fusion constructs produced a functional lacZ gene when tested in cell culture, as demonstrated by X-Gal staining of transfected cells and ß-gal activity assays. Apart from having an intact coding sequence, lacZ protein should be intracellular to be active. In the HaPrP-lacZ fusion proteins produced here, we assume that the HaPrP region of the protein was extracellular, attached to the membrane either by a GPI anchor, as normally expected (see 1.7) or, in case the GPI addition reaction was inhibited by the presence of lacZ in the fusion protein, by the short transmembrane sequence that serves as the signal for GPI addition. The lacZ part of the protein should remain intracellular, maybe attached to the membrane by the transmembrane part of PrP, or released as a soluble protein in the cell (by unknown mechanism) and therefore active. This fact enabled the comparison of translation efficiency between the constructs.

The results shown here suggest that there are no major differences in translation efficiency produced by the use of the different 5' UTR fragments. However, pE1,2HaPßgeo appears to translate slightly better than the other two constructs and the construct that has only 10 nucleotides of HaPrP exon 2 as a 5' UTR, translates worse. Any increase or decrease in translation efficiency observed is less than 2-fold.

These data suggest that even if exon 1 contains some regulatory elements they are probably rather weak, because the differences in translation efficiency are not significant.

The possibility that regulatory elements in the 5' UTR are active when HaPrP sequences alone are present, or an interaction between 5' and 3' HaPrP UTR is crucial for regulation of translation, cannot be excluded (see also conclusions in 3.4.3). These issues are investigated in chapter 4.

CHAPTER 4

The effect of 3' and 5' untranslated sequences on the translation of hamster PrP

4.1 Introduction

The role of 3' UTR sequences on translation was discussed in section 3.1. Although it has been postulated that the HaPrP 3' UTR might have an effect on the translation efficiency of HaPrP, it does not appear to have a significant effect on lacZ translation (section 3.4.3). Similar observations were obtained when the effect of different 5' UTR sequences of HaPrP on lacZ translation was tested (section 3.5.3).

It was decided that the effect of these sequences on the translation of HaPrP should be investigated. Three constructs based on pBluescriptIIKS- were initially generated (section 4.2) and tested in an *in vitro* translation system in order to assess whether these HaPrP fragments were translatable and whether there were significant differences in the amounts of protein produced (section 4.3). These fragments were subcloned into the vector pCEP4 (appendix 2) and used for expression experiments in cell culture. Problems with the production and/or the detection of HaPrP protein were encountered (section 4.4.1). The use of different cell lines and different protein analysis techniques was investigated. Further constructs, based on pEE6hCMV vector, were generated (section 4.5) and tested in cell culture (section 4.6). A construct containing the same HaPrP fragment as that used in the transgenic construct was generated and tested in parallel. Latterly a detection method for HaPrP protein has been established. This made possible the quantification of translation efficiencies of the different constructs (section 4.6).

4.2 Generation of pBluescriptIIKS-HaPrP and pCEP4-HaPrP constructs.

The different HaPrP fragments (Figure 4.2.1) were obtained either by PCR from hamster brain genomic DNA, or by RT-PCR from hamster brain total RNA, using the proofreading DNA polymerase UlTma (Perkin Elmer). All fragments were initially cloned in pBluescriptIIKS- and were fully sequenced to ensure that no mutations had been introduced during the PCR step. The fragment HaP contains the 5' UTR of exon 2 (10 nucleotides, see Figure 3.5.2) and the same 3' as the HaPrP fragment used for the generation of transgenics (168 nucleotides of the 3' UTR, see also section 2.3.1). The forward primer used to amplify by PCR this fragment was 5'ex.2.b (appendix 1) and introduced an XhoI site. The reverse primer, 3'UTR.3 (appendix 1) is similar to oligo 3'HaP, used for PCR amplification of the HaPrP fragment in the transgenic construct, but includes a BamHI site. The HaPU fragment has the same 5' end as the HaP fragment but includes the 3'UTR of HaPrP (1.2 kb). The forward primer was 5'ex.2.b (appendix 1) and the reverse primer, 3'UTR.2 (appendix 1) introduced a BamHI site. The HaEPU fragment represents the complete cDNA HaPrP fragment and was obtained by RT-PCR from hamster brain RNA. It has the same 3' end as the HaPU fragment but includes exon 1 (60 nucleotides, see Figure 3.5.2) at the 5' end. The forward primer, 5'ex.1.b (appendix 1), introduced an XhoI site. The reverse primer was 3'UTR.2, used also for PCR amplification of the HaPU fragment. The three XhoI-BamHI fragments were cloned into the pBluescriptIIKS- (Figure 4.2.1) before subcloning into pCEP4 (Figure 4.2.2).

4.3 *In vitro* translation of HaPrP constructs

As mentioned in section 4.1, in order to test whether the different HaPrP segments were translatable and to compare approximately the amount of protein that was produced, a rabbit reticulosyte lysate (RRL) *in vitro* translation system was used (section 7.25). RNA was prepared *in vitro* with T3 RNA polymerase (section 7.10) from the pBlueHaP, pBlueHaPU and pBlueHaEPU constructs (Figure 4.2.1) after linearisation

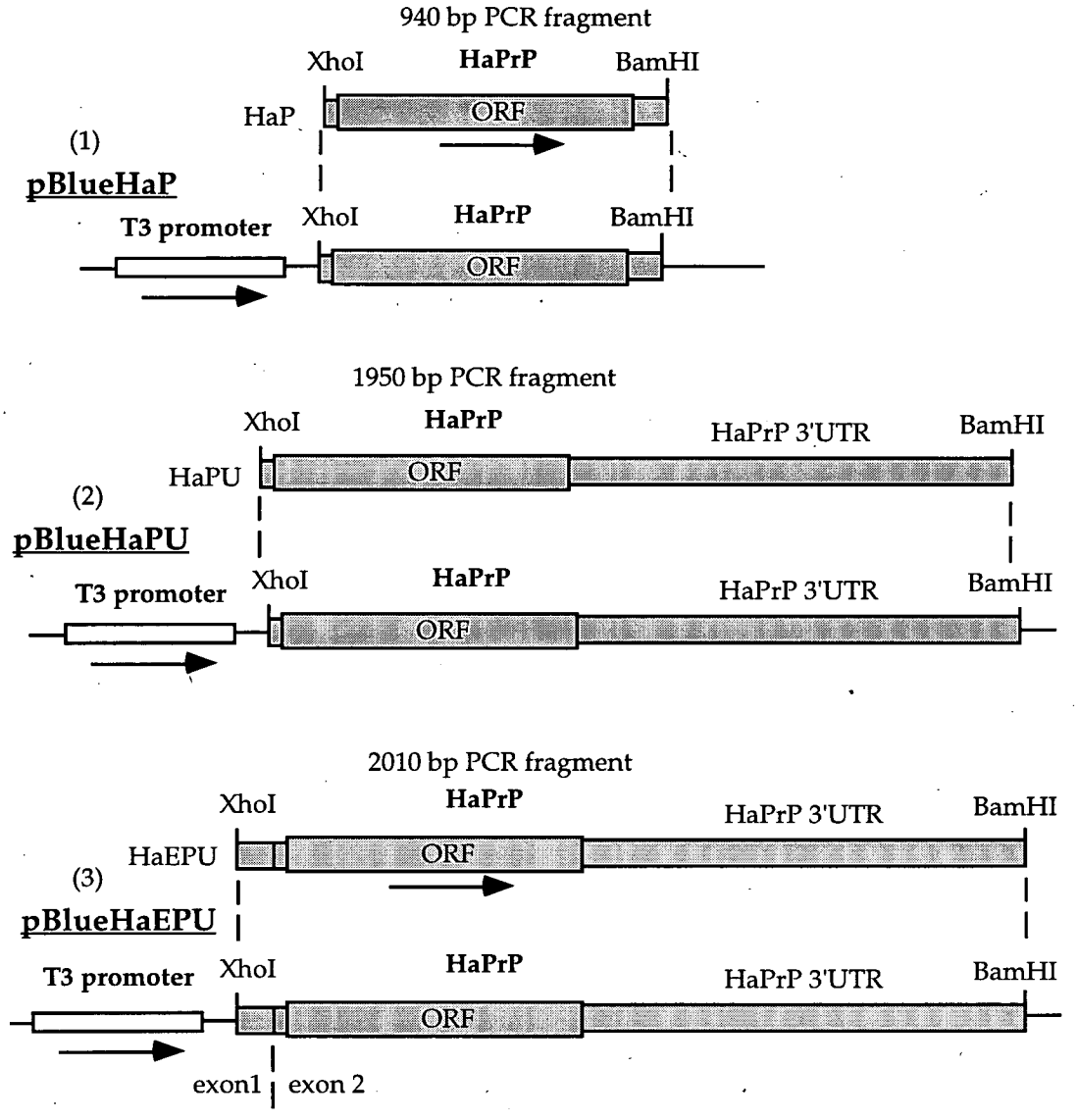


Figure 4.2.1 Cloning of HaPrP PCR fragments in pBluescriptIIKS-

(1) Cloning of the 940 bp HaP PCR fragment in pBluescriptIIKS- to generate pBlueHaP.

(2) Cloning of the 1950 bp HaPU PCR fragment in pBluescriptIIKS- to generate pBlueHaPU.

(3) Cloning of the 2010 bp HaEPU PCR fragment in pBluescriptIIKS- to generate pBlueHaEPU.

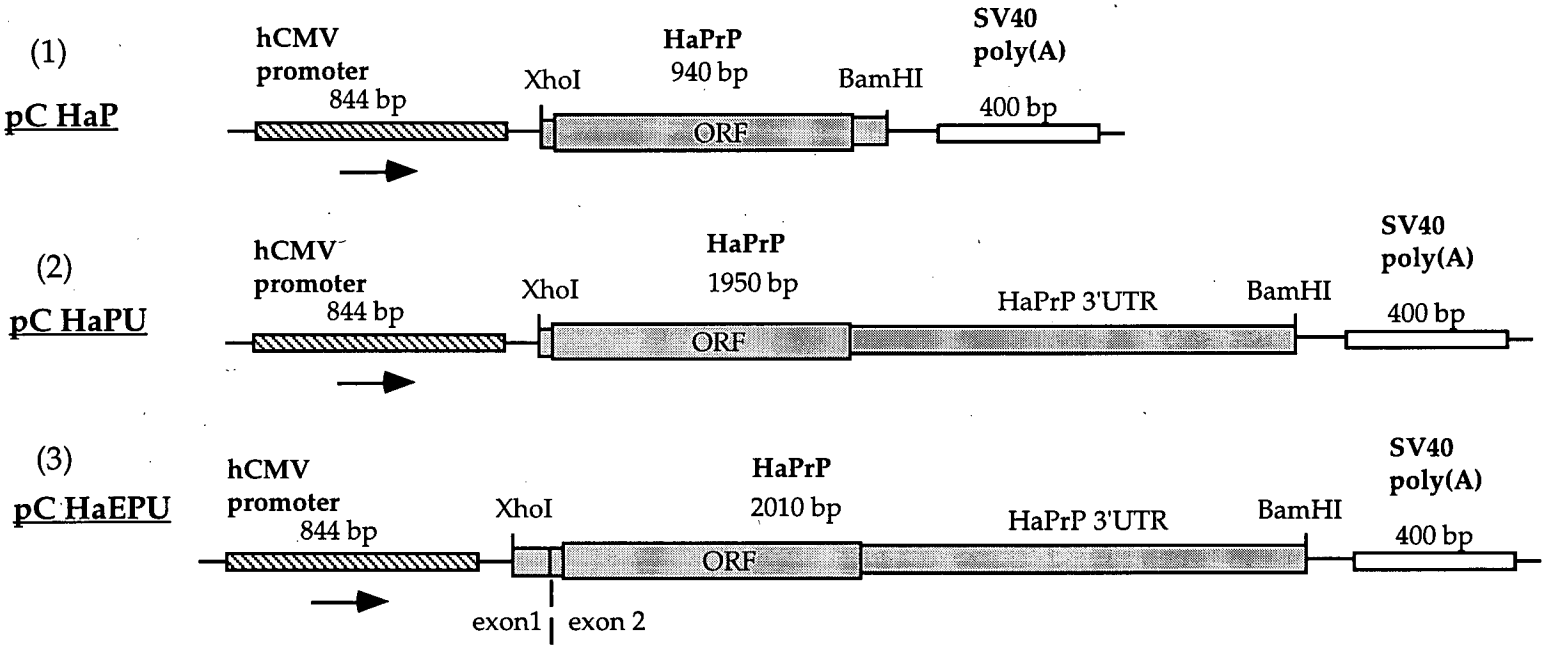


Figure 4.2.2 Generation of pCEP4-HaPrP constructs

(1) Cloning of the 940 bp HaPrP fragment in pCEP4 to generate the construct pC HaP.

(2) Cloning of the 1950 bp HaPrP fragment in pCEP4 to generate the construct pC HaPU.

(3) Cloning of the 2010 bp HaPrP fragment in pCEP4 to generate the construct pC HaEPU.

Figure 4.3.1 *In vitro* translation and immunoprecipitation of pBluescriptIIKS- HaPrP constructs

A. Translation of pBlueHaP and pBlueHaPU constructs in the Promega RRL.

500 ng of *in vitro* produced RNA (section 7.8) were used for each reaction and half of the reaction was used for SDS-PAGE (section 7.21.3).

1. translation of pBlueHaP RNA
2. translation of pBlueHaPU RNA
3. negative control: no RNA

B. Translation of pBlueHaP and pBlueHaEPU constructs in the Gibco-BRL RRL.

500 ng of *in vitro* produced RNA (section 7.8) were used for the reactions and half of each reaction was used for SDS-PAGE (section 7.21.3) in lanes 1 and 2.

500 ng of CAT RNA (provided by the manufacturers) was used in a translation reaction and a quarter was used for SDS-PAGE in lane 5.

1. translation of pBlueHaEPU RNA
2. translation of pBlueHaP RNA

The remaining of the reaction shown in lane 1 (translation of pBlueHaEPU) was split in two samples and each was used for immunoprecipitation (ip) with the 3F4 ab (lanes 3 and 4).

3. ip of pBlueHaEPU-produced HaPrP with 3F4 ab 1:500
4. ip of pBlueHaEPU-produced HaPrP with 3F4 ab 1:250
5. control reaction: CAT (24 kD)

Immunoprecipitation of *in vitro* translated pBlueHaPU construct in the Promega (**C**) and the Gibco-BRL (**D**) RRL. 500 ng of *in vitro* produced RNA (section 7.8) were used for each reaction and the whole reaction was used for immunoprecipitation (ip) with the 3F4 ab. 1 μg of luciferase, or CAT RNA provided by the manufacturers, was also translated and half of the reactions were used for SDS -PAGE.

C.
1. control reaction: luciferase (68 kD)
2. negative control reaction: no RNA
3. ip of pBlueHaPU-produced HaPrP with 3F4 ab 1:200

D.
1. ip of pBlueHaPU-produced HaPrP with 3F4 ab 1:400
2. control reaction: CAT (24 kD)
3. negative control reaction: no RNA

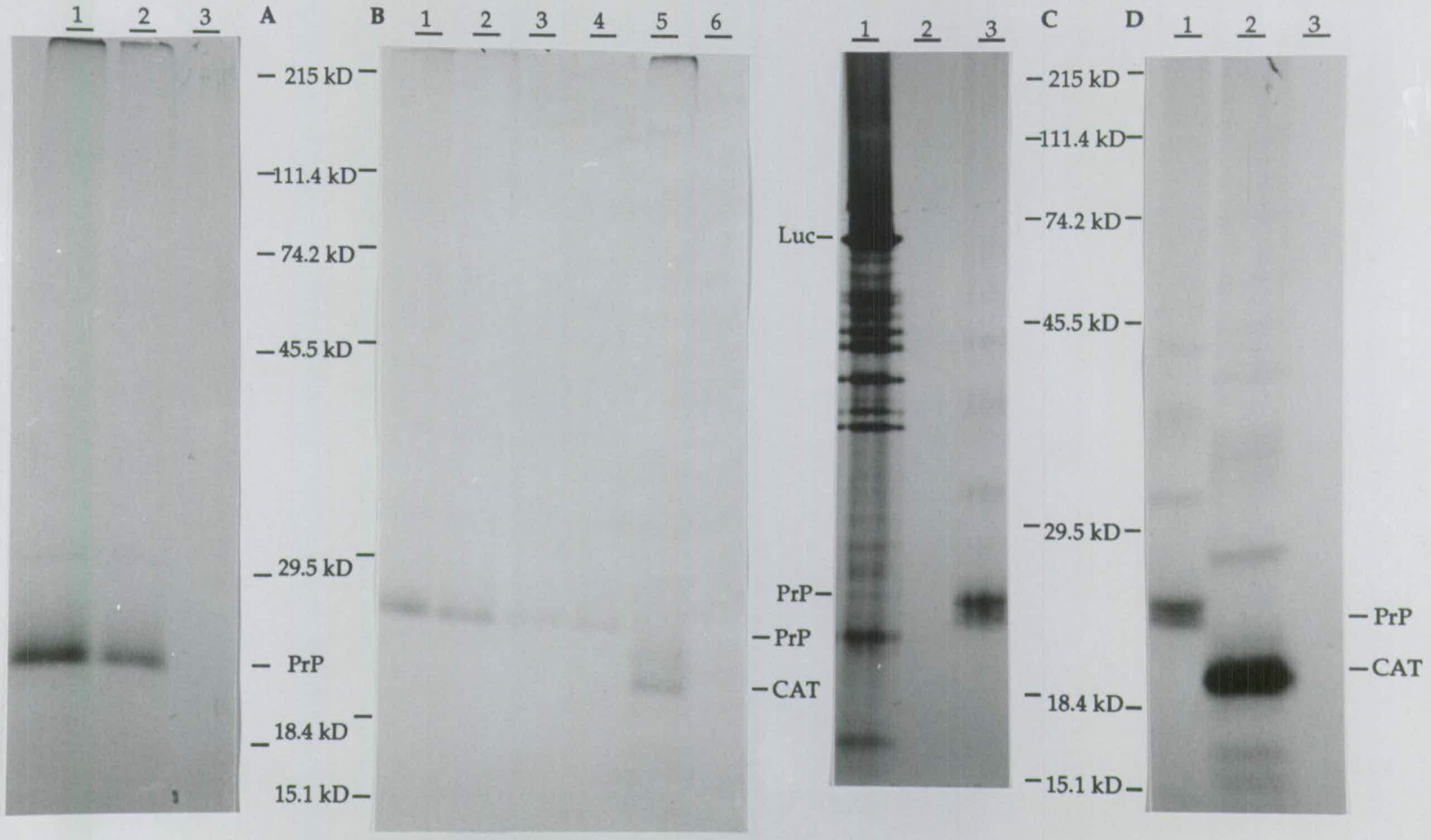

1
2
3
A
B
1
2
3
4
5
6
215 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
PrP
18.4 kD
15.1 kD
1
2
3
Luc
PrP
PrP
CAT
C
D
1
2
3
215 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
15.1 kD
1
2
3
PrP
CAT

figure 4.3.1

with BamHI (which cuts in all constructs just beyond the 3' end of the HaPrP fragments). After purification of the RNA produced by *in vitro* transcription and quantification, approximately the same amount of RNA was used from each construct for translation in the RRL. The three constructs appeared to produce HaPrP protein, which is unglycosylated and therefore 26-27 kD in size (Figure 4.3.1). The protein produced was also immunoprecipitated (ip, section 7.23) with the monoclonal antibody (ab) 3F4 (Kascsak *et al.*, 1987) (Figure 4.3.1B,C,D). Also, a translation reaction with control CAT, or luciferase RNA (provided by the RRL manufacturers, section 7.25) was sometimes performed in parallel with the other reactions (4.3.1C,D). The fainter bands appearing in most lanes above the main protein band are probably multimers of the protein and those bands below are presumed to be proteolytic products.

These results demonstrate that the three different HaPrP fragments are able to produce HaPrP protein and there is no major difference in the amount produced. Immunoprecipitation of the *in vitro* translated PrP protein was performed. Establishment of this method was crucial for the detection of HaPrP protein in cell culture and it was used instead of Western blot (section 4.6).

4.4 Translation of pCEP4-HaPrP constructs in cell culture

4.4.1 Initial attempts: difficulties in detecting HaPrP protein

The three pCEP4 based constructs pC HaP, pC HaPU and pC HaEPU, were initially tested for their translation efficiency in cos7 cells. Transient transfections were performed by the calcium phosphate method (section 7.29.5). Half of the harvested transfected cells was used for RNA extraction and the rest for protein extraction. The results of nine transfection experiments are shown in Figure 4.4.1. Northern blot analysis (Figure 4.4.1A) showed that it was possible to achieve production of substantial amounts of mRNA by the transfected constructs, but PrP protein was not detectable by Western blot analysis (Figure 4.4.1B,C). These data indicated that either the mRNA produced was not translated efficiently and/or that the Western blot analysis was

Figure 4.4.1

A. Northern blot analysis transiently transfected cos7 cells

The probe used was the HaP fragment (HaPrP ORF-containing 940 bp fragment, see Figure 4.2.1)

	cells	construct
1.	cos7	pC HaP
2.	cos7	pC HaEPU
3.	cos7	pC HaPU
4.	cos7	pC HaP
5.	cos7	pC HaEPU
6.	cos7	pC HaEPU
7.	cos7	pC HaPU
8.	mouse brain	
9.	cos7	pCEP
10.	N2a	pCEP (underloaded)

B and **C** Western blot analysis of transiently transfected cos7 cells

B. Probed with the mouse monoclonal HaPrP specific ab 3F4.

C. probed with the rabbit polyclonal anti-PrP ab 1B3.

The samples on both blots are in the same order.

	cells	construct
1.	size markers	
2.	cos7	pC HaP
3.	cos7	pC HaEPU
4.	mouse brain	
5.	cos7	pCEP
6.	N2a	pCEP

The protein extracts used in lanes 2 and 3 correspond to the RNA samples from transfected cell lines shown in lanes 1 and 2, respectively, in the Northern blot analysis (**A**), and the protein extracts in lanes 5 and 6 correspond to lanes 9 and 10, respectively, in **A**. No Western blot analysis is shown for the rest transiently transfected cell lines analysed by Northern blot in **A**, because the amount of RNA produced by the constructs in these cell lines was very low.

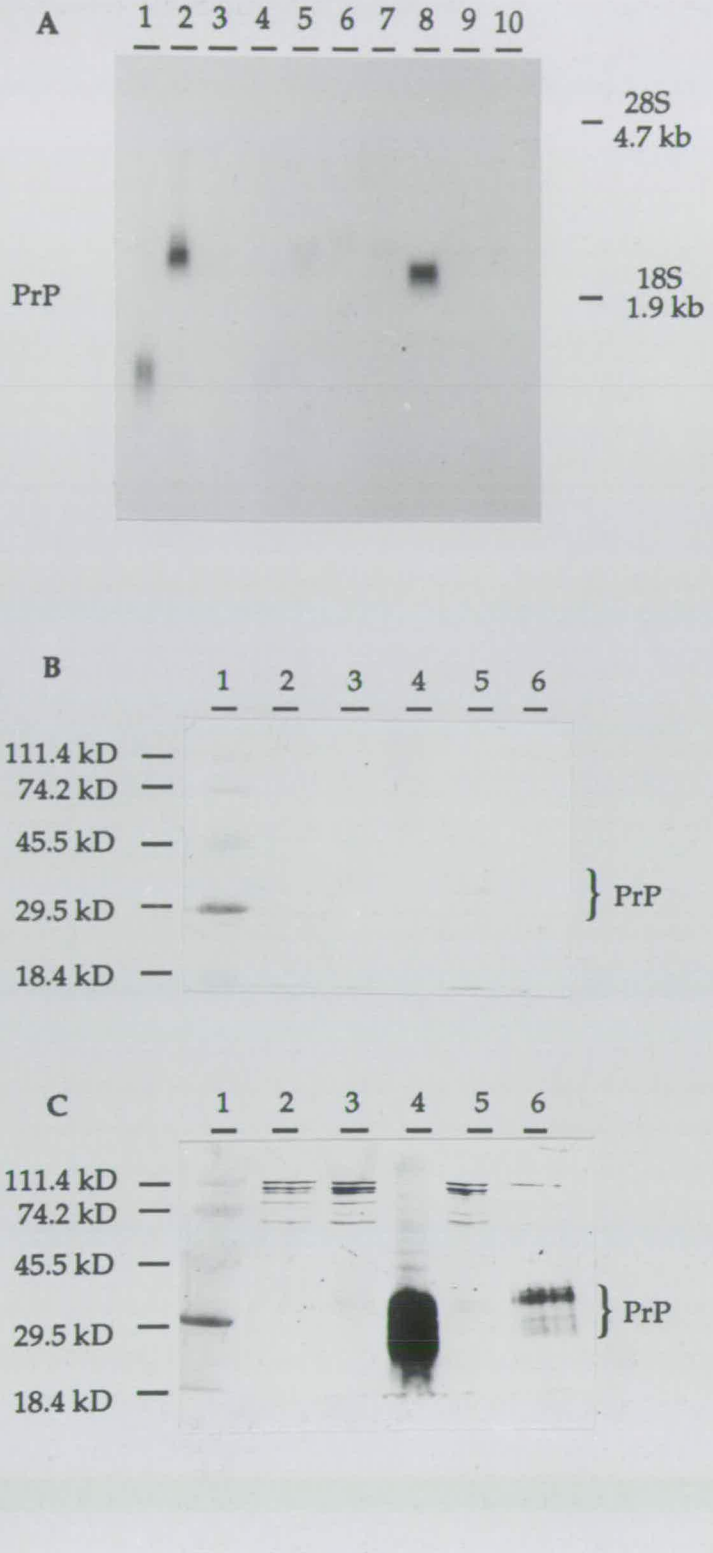

A
1 2 3 4 5 6 7 8 9 10
28S
4.7 kb
PrP
18S
1.9 kb
B
1 2 3 4 5 6
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1 2 3 4 5 6
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 4.4.1

not sensitive enough. Also, endogenous PrP mRNA was present in cos7 cells but PrP protein was not detectable, in contrast to N2a cells (mouse neuroblastoma), suggesting that the PrP protein production/detection problem could be due to the use of cos7 cells. Previous studies have shown that although cos7 are able to express PrP, the protein does not seem to be normally processed and substantial amounts were found to be secreted into the medium instead of being retained on the plasma membrane (Scott *et al.*, 1988), suggesting that probably cos7 cells were not the best system for studying PrP expression. N2a cells are now used extensively for expression of PrP (section 1.12.2). The difference in translation efficiency regarding PrP between cos7 and N2a is possibly due to the fact that N2a are of neuronal origin while cos7 are kidney-derived. Similar observations were made with different mouse tissues: brain translates PrP mRNA much more efficiently than any other tissue (see also section 2.8).

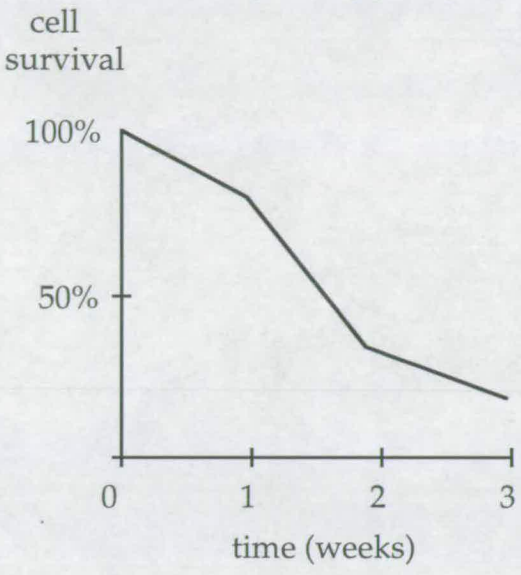
Several attempts to transfect N2a cells by the calcium phosphate method (which gave high percentage of transfected cells when performed on cos7 cells), failed repeatedly. Transfection using lipofectin was also attempted without success, although different concentrations of DNA and amounts of lipofectin were tried (data not shown). Electroporation was also applied, giving better results from the other methods. Table 4.4.1 presents some of the different parameters tested for electroporation of N2a cells. Electroporation was not without problems and often produced extensive cell death. It was determined that stably transfected lines should be generated. The fact that the pCEP4 construct included the gene for hygromycin resistance facilitated the production of stably-transfected N2a cell lines. Stable lines were also produced with cos7 (using the calcium phosphate method) so as to continue the comparison between the two cell lines. N2a cells were electroporated with linearised pCEP4-HaPrP constructs (using the single ClaI site beyond the SV40 poly(A) sequence, see pCEP4 appendix 2). 72 hours after transfection the cells underwent selection in medium containing hygromycin for approximately three weeks before they were analysed. A titration of the amount of hygromycin that should be used was initially determined on both N2a and cos7 as shown in Figure 4.4.2. After the selection period, aliquots were harvested and used for RNA and protein analysis. Stable cell lines were maintained in medium containing

No of cells	media 500 µl	Voltage (V)	Capaci-tance (µF)	amount of DNA	~survival%	~transfected%
$2X10^6$	PBS	150	500	10 µg	60%	>1%
$2X10^6$	PBS	150	960	10 µg	60%	>1%
$4X10^6$	optimem	250	500	10 µg	30%	>1%
$4X10^6$	optimem	250	960	10 µg	20%	>1%
$3X10^6$	optimem	450	500	10 µg	5%	1-5%
$3X10^6$	optimem	450	960	10 µg	1-5%	1-5%
$3X10^6$	optimem	450	500	30 µg	5%	1-5%
$3X10^6$	optimem	450	960	30 µg	1-5%	1-5%
$2X10^6$	PBS	450	500	10 µg	5%	1-5%
$2X10^6$	PBS	450	960	10 µg	1-5%	5%
$3X10^6$	PBS	450	500	30 µg	5%	5%
$3X10^6$	PBS	450	960	30 µg	1-5%	5-10%

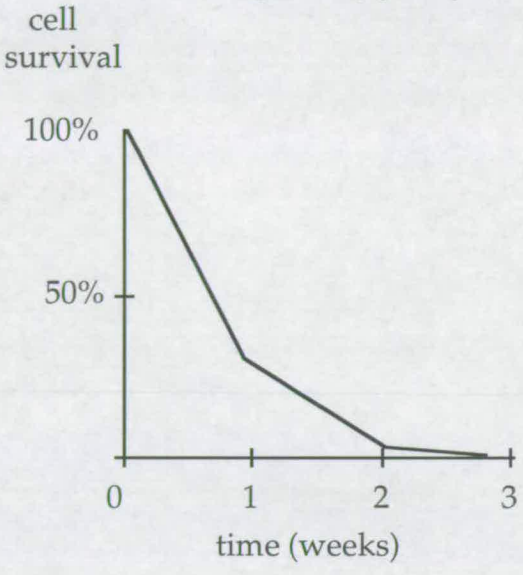
Table 4.4.1 Electroporation parameters tested on N2a cells

cos7

1a. 200 µg/ml hygromycin

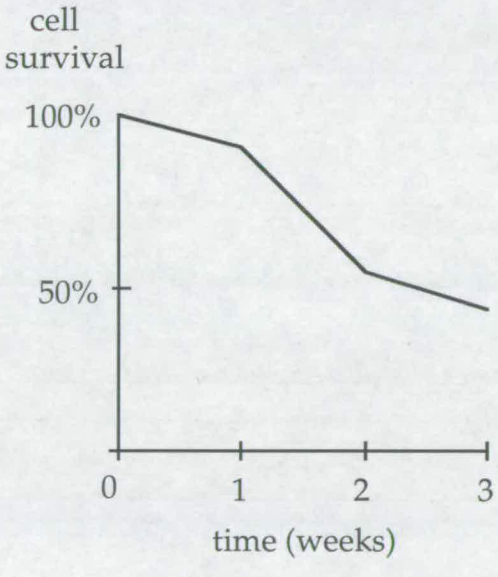


1b. 400 µg/ml hygromycin



N2a

2a. 400 µg/ml hygromycin



2b. 600 µg/ml hygromycin

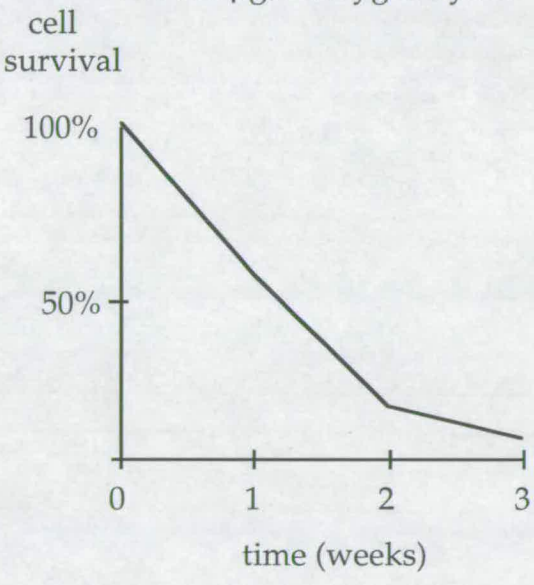


Figure 4.4.2 Hygromycin selection of cos7 and N2a cells

1a. Survival of cos7 cells in 200 µg/ml hygromycin.

1b. Survival of cos7 cells in 400 µg/ml hygromycin.

2a. Survival of N2a cells in 400 µg/ml hygromycin.

2b. Survival of N2a cells in 600 µg/ml hygromycin.

Figure 4.4.3

A. Northern blot analysis of stable cell lines

The probe used was the HaP fragment (940 bp, see Figure 4.2.1)

	cells	construct
1.	mouse brain	
2.	-	
3.	N2a	
4.	cos7	
5.	cos7	pC HaP
6.	cos7	pC HaEPU
7.	N2a	pC HaP
8.	N2a	pC HaPU
9.	N2a	pC HaEPU

B. and **C.** Western blot analysis of stable cell lines

B. Probed with the mouse monoclonal HaPrP specific ab 3F4

C. Probed with the rabbit polyclonal anti-PrP ab 1B3

The samples are in the same order on both blots.

	cells	construct
1.	N2a	

Background control: only second ab was used (in B: anti-mouse IgG, in C: anti-rabbit IgG)

2.	size markers	
3.	cos7	pC HaP
4.	cos7	pC HaEPU
5.	N2a	
6.	N2a	pC HaP
7.	N2a	pC HaEPU
8.	mouse brain	

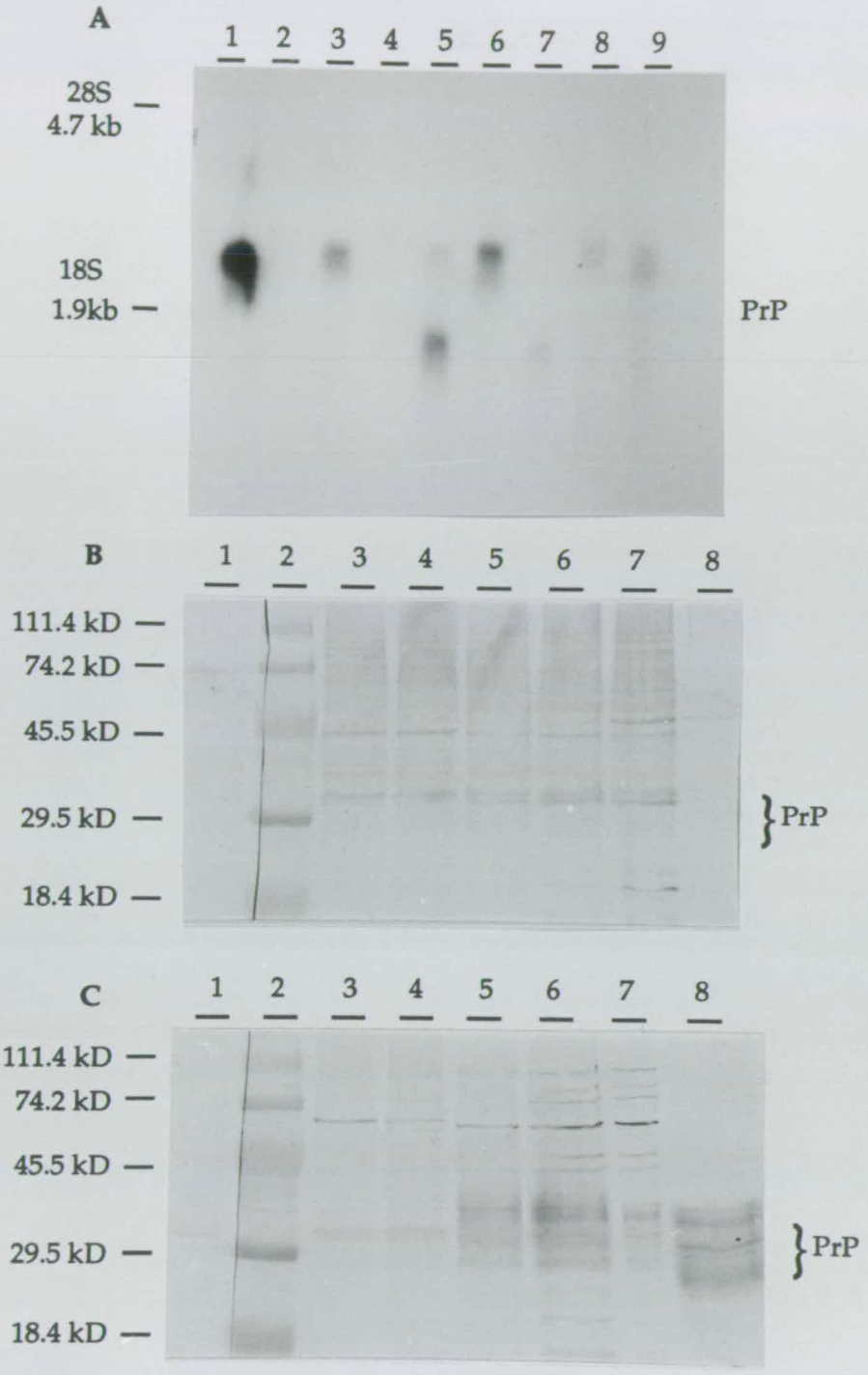

A
1 2 3 4 5 6 7 8 9
28S
4.7 kb
18S
1.9kb
PrP
B
1 2 3 4 5 6 7 8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1 2 3 4 5 6 7 8
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 4.4.3

hygromycin. No individual colonies were picked at the end of the selection period. In each pool examined there were approximately 20-30 colonies. Three N2a and three cos7 pools were made with the three constructs: pC HaP, pC HaPU, and pC HaEPU. The mRNA and protein analysis from some of the stable cell lines is shown in Figure 4.4.3. These data demonstrated that the stable cell lines show low mRNA expression and no detectable protein on a Western blot. Also, Western blot analysis appeared to give very high background, especially with the 3F4 antibody.

Another construct pEE6hCMVneo-HaPrP (appendix 2, see also section 3.5.1) that was shown previously to express HaPrP protein in cell culture (Dr A. Bennett and Dr L. Wightman personal communication; pEE6hCMV-HaPrP was agenerous gift from Dr L. Wightman) was used as a positive control. This construct is based on pEE6hCMVneo construct (Celltech) which contains a fragment of the human cytomegalovirus (CMV) enhancer-promoter and includes the 5' UTR of the CMV immediate early gene with the leader intron. The HaPrP fragment (800 bp) was cloned as a HindIII-EcoRI fragment in the polylinker of this construct and contained only the HaPrP ORF (from the ATG start codon to TGA stop codon) and had the 'optimal' initiation of translation consensus sequence upstream of the ATG (see also Figure 3.5.2). When this construct was tested by transient transfection in cos7 cells and was compared for mRNA and protein expression with the stable lines generated, it appeared to produce much higher amounts of mRNA and some HaPrP protein (Figure 4.4.4). However, the HaPrP protein produced did not give exactly the expected size or pattern (Figure 4.4.4B,C, lanes 5, 6, 7) indicating that it may not be correctly processed. For the mRNA analysis presented in Figure 4.4.4A, hybridisation with the S26 sequence (mouse ribosomal protein, gift from Dr B. Davies) was used as a loading control, although the hybridisation signal obtained from N2a cells is much stronger than that from cos7, because N2a cells are of mouse origin. Therefore loading comparisons between N2a and cos7 cells are based on ethidium bromide staining.

Figure 4.4.4

A. Northern blot analysis transfected cell lines

The probe used was the HaPrP fragment (940 bp, see Figure 4.2.1)

	cells	transfection	construct
1.	cos7	transient	pCEP
2.	cos7	stable	pC HaP
3.	cos7	stable	pC HaPU
4.	cos7	stable	pC HaEPU
5.	cos7	transient	pE HaKo
6.	N2a		
7.	N2a	stable	pC HaP
8.	N2a	stable	pC HaPU
9.	N2a	stable	pC HaEPU
10.	mouse brain		

B. and **C.** Western blot analysis of transfected cell lines

B. Probed with the mouse monoclonal HaPrP specific ab 3F4

C. Probed with the rabbit polyclonal anti-PrP ab 1B3

The samples are in the same order on both blots.

	cells	transfection	construct
1.	cos7	transient	pCEP
2.	cos7	stable	pC HaP
3.	cos7	stable	pC HaPU
4.	cos7	stable	pC HaEPU
5.	cos7	transient	pE HaKo
6.	cos7	transient	pE HaKo
7.	cos7	transient	pE HaKo
8.	N2a		
9.	N2a	stable	pC HaP
10.	N2a	stable	pC HaPU
11.	N2a	stable	pC HaEPU
12.	mouse brain		

From the transiently transfected cells the protein extract on lane 5 corresponds to the RNA shown in A lane 5. No RNA data are shown corresponding to the protein samples 6 and 7.

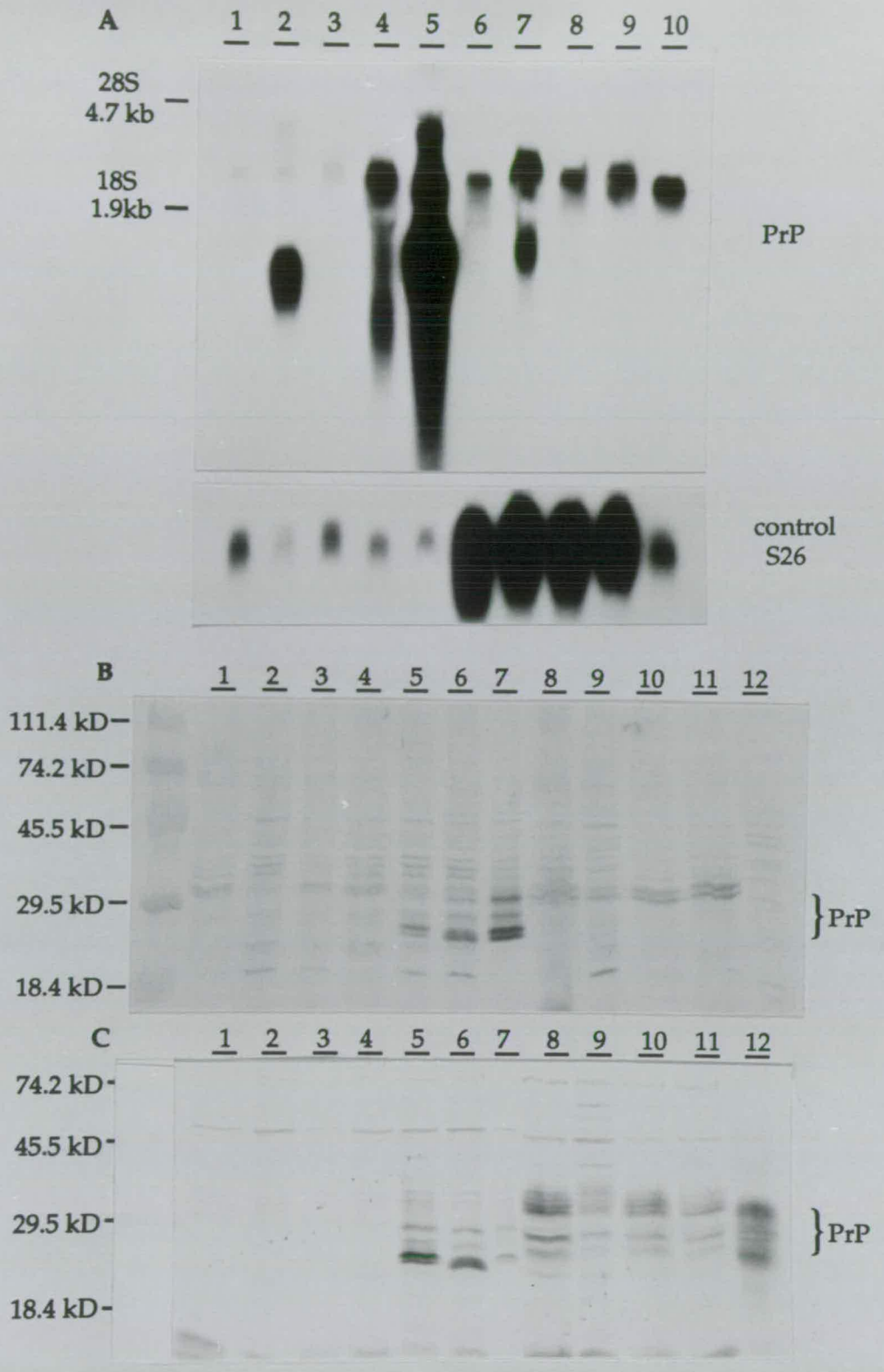

A
1 2 3 4 5 6 7 8 9 10
28S
4.7 kb
18S
1.9kb
PrP
control
S26
B
1 2 3 4 5 6 7 8 9 10 11 12
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1 2 3 4 5 6 7 8 9 10 11 12
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 4.4.4

4.4.2 Conclusions

The generation of the stable lines did not help in elucidating the problems encountered with the translation and/or detection of HaPrP protein because they produced very low amounts of mRNA and no detectable protein.

It was postulated that the difficulty in transfecting N2a cells might have been specific to the N2a substrain employed. Therefore N2a cells were obtained from a different source (ICN) and evaluated for their transfection efficiency.

The pEE6hCMVneoHaPrP construct used as a control, had a fragment of the human CMV that included an intron (not present in the human CMV promoter fragment of pCEP4). Also the HaPrP fragment in that construct did not have the original initiation of translation consensus sequence but the 'optimal' consensus sequence (see Figure 3.5.2). The importance of the intron and the initiation of translation consensus sequences have been discussed in section 3.1. It was decided that the effect of the hCMV intron and the different consensus sequence should be tested on the translation of PrP. More constructs based on pEE6hCMVneo were generated as presented in section 4.5. These constructs, together with the pCEP4-based constructs, were tested by transient transfection in both cos7 and N2a cells (section 4.6). Quantification of the translation results became possible after establishing an efficient transient transfection method for N2a cells and efficient PrP protein detection method (immunoprecipitation), (section 4.6).

4.5 Generation of pEE6hCMVneo-HaPrP constructs

The pEE6hCMVneo vector (appendix 2, see also section 4.4.1) was used for subcloning different HaPrP fragments previously cloned in pCEP4 (constructs pC HaP and pC HaEPU). The HaP and the HaEPU fragments were cloned in pEE6hCMVneo to generate pE HaP and pE HaEPU constructs as shown in Figures 4.5.1 and 4.5.2. The same cloning strategy was followed for both fragments. The EcoRI linker used was

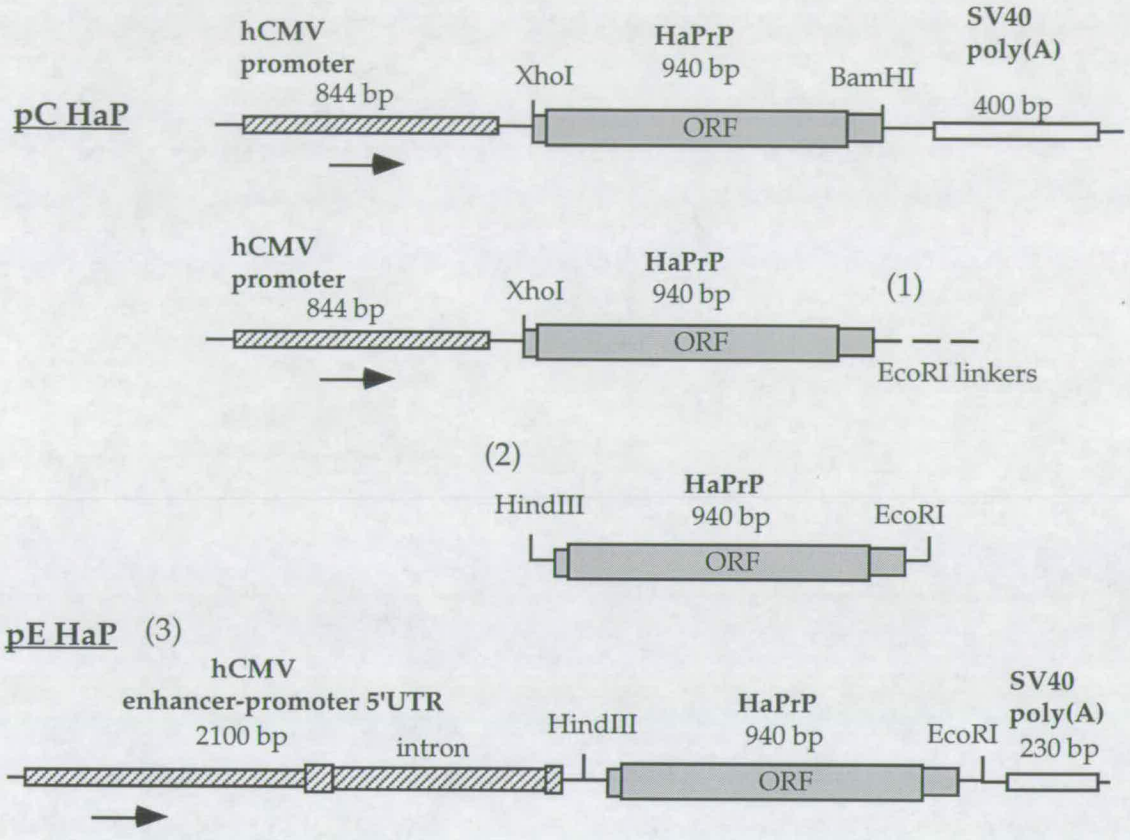


Figure 4.5.1 Generation of pE HaP construct

(1) pC HaP was digested with BamHI and EcoRI linkers were added, but the final digestion step with EcoRI was omitted. The fragment was purified through a 'Wizzard' column to avoid the remaining un ligated EcoRI linkers.

(2) The HaPrP fragment was digested with XhoI and HindIII adaptors were ligated. EcoRI digestion was applied to remove the EcoRI linker concatamers.

(3) HindIII-EcoRI HaPrP fragment was eventually cloned in pEE6hCMVneo to generate pE HaP.

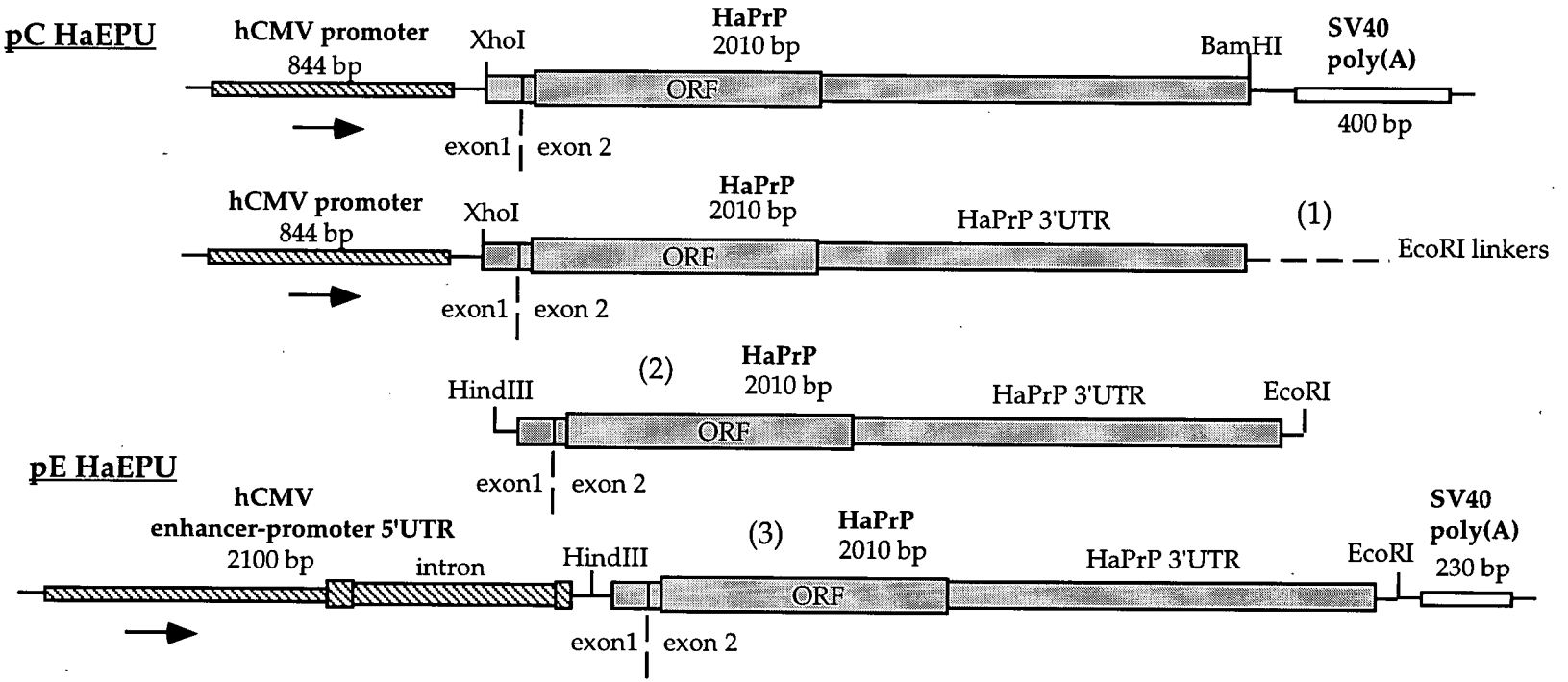


Figure 4.5.2 Generation of pE HaEPU construct

(1) pC HaEPU was digested with BamHI and EcoRI linker was added, but the final digestion step with EcoRI was ommited. The fragment was purified through a 'Wizzard' column to avoid the remaining un ligated EcoRI linker.

(2) The HaPrP fragment was digested with XhoI and HindIII adaptors were ligated. EcoRI digestion was applied to remove the EcoRI linker concatamers.

(3) HindIII-EcoRI HaPrP fragment was eventually cloned in pEE6hCMVneo to generate pE HaEPU.

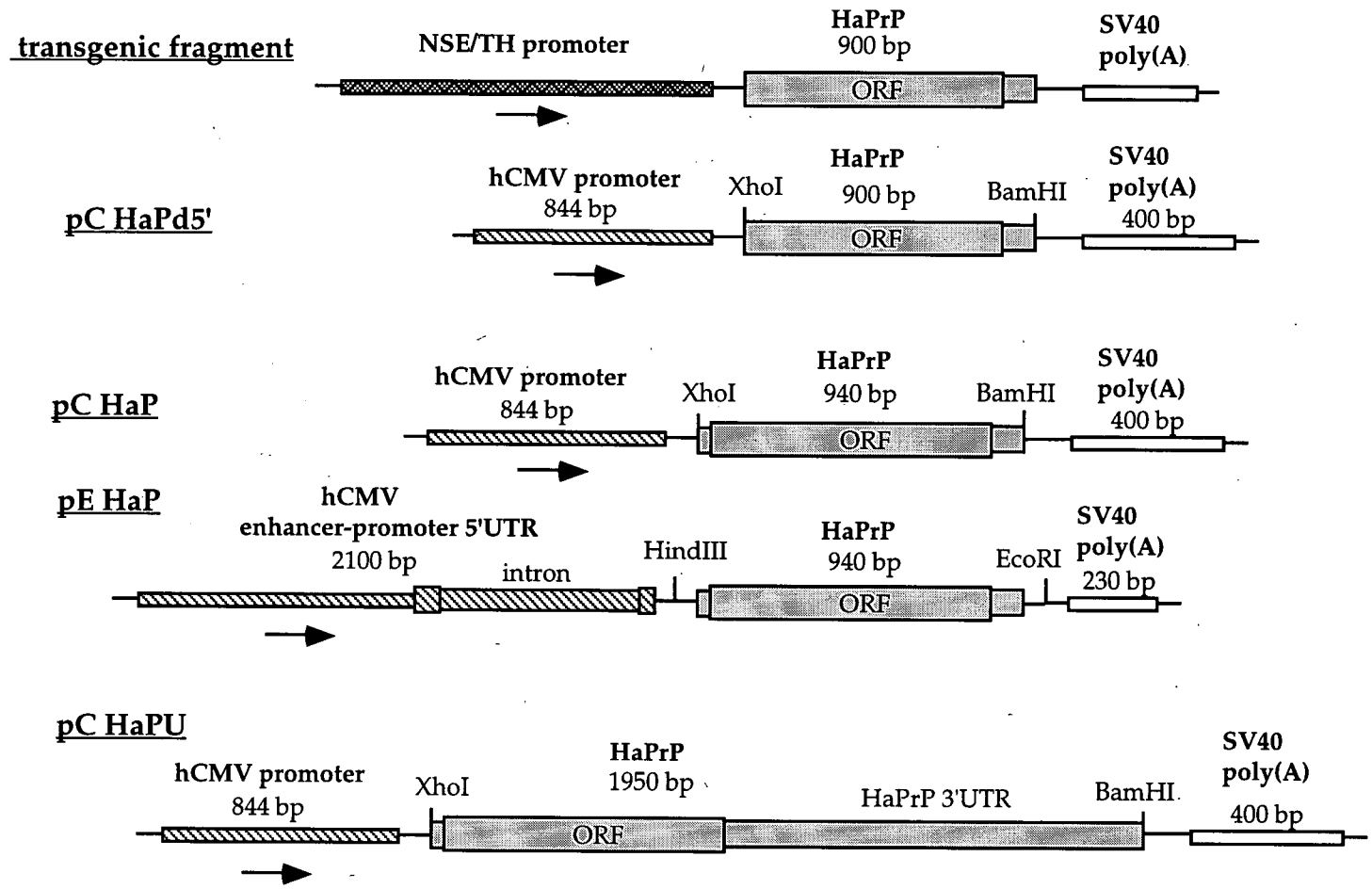


Figure 4.5.3 Generation of constructs tested in cell culture (a)

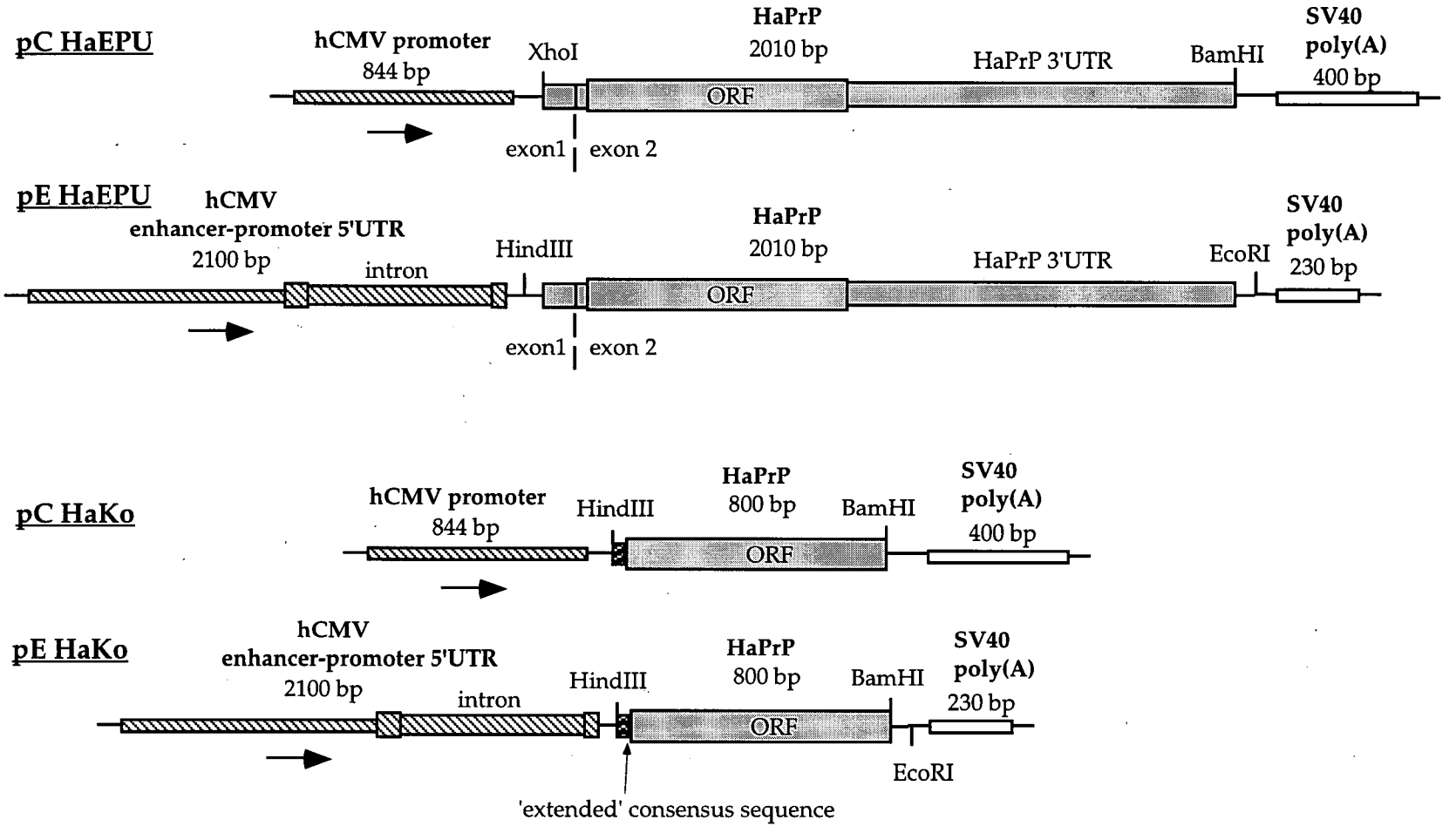


Figure 4.5.4 Generation of constructs tested in cell culture (b)

oligo EcoRIl. (appendix 1) and the HindIII adaptors were oligos Hind.ad.1 and Hind.ad.2 (appendix 1).

It was also decided to subclone the HindIII-BamHI HaPrP fragment (800 bp) that was cloned in pEE6hCMVneoHaPrP construct, in pCEP4 to generate the construct pC HaKo. The PEE6hCMVneoHaPrP construct was renamed pE HaKo for simplicity.

Testing the translation efficiency of the HaPrP fragment (900 bp) used initially for the generation of transgenics in cell culture was considered important. This fragment was initially cloned as a HindIII-EcoRI fragment in the polylinker of pBluescriptIIKS-. The XhoI and BamHI sites of the construct (Figure 2.3.1) could be used to excise that fragment and clone it between the XhoI and the BamHI sites of pCEP4 to generate the construct pC HaPd5'. All the constructs generated are presented in Figures 4.5.3 and 4.5.4.

4.6. Translation of pCEP-HaPrP and pEE6hCMVneo-HaPrP constructs in cell culture

4.6.1 Comparison of translation efficiency between the constructs

The transfection efficiency of the more recent aliquot of N2a cells was tested. Different transfection methods were used and compared to those of the N2a cells used for all previous transfections. These cells responded in a different way and also showed morphological differences. The calcium phosphate method gave higher transfection efficiency and less cell death than the two other methods applied, electroporation and lipofection (data not shown). This method enabled quick and efficient testing of the constructs by transient transfection and therefore helped to overcome the problems encountered with the stable transfections (section 4.4.2).

Efforts also concentrated on establishing an efficient way for detecting PrP protein. Immunoprecipitations (ip, section 7.23) from ^{35}S-labeled (section 7.22.1), transfected cells (section 7.29.5) provided a solution to the detection problems encountered when Western blot analysis was applied. This method allowed the detection of small amounts of PrP protein and reduced the background.

Figure 4.6.1

Immunoprecipitation of transfected cell lines with the 3F4 mouse monoclonal Ha PrP specific ab.

	cells	transfection	construct
1.	N2a	transient	pCEP

(immunoprecipitated with the rabbit polyclonal anti-PrP ab 1A8).

2.	N2a	transient	pCEP
3.	N2a	transient	pE HaKo
4.	N2a	transient	pE HaKo
5.	N2a	stable	pC HaP
6.	N2a	transient	pC HaP
7.	N2a	transient	pC HaEPU
8.	cos7	transient	pCEP
9.	cos7	transient	pE HaKo
10.	cos7	transient	pE HaEPU

Figure 4.6.2

Pulse-chase of transiently transfected N2a and cos7

N2a and cos7 were transiently transfected with pC HaEPU construct and the protein extract was immunoprecipitated after 2 h pulse and 0, 4 and 8 h chase.

A. pulse-chase of N2a cells

1. 0 h chase
2. 4 h chase
3. 8 h chase

B. pulse-chase of cos7 cells

1. 8h chase
2. 4 h chase
3. 0 h chase

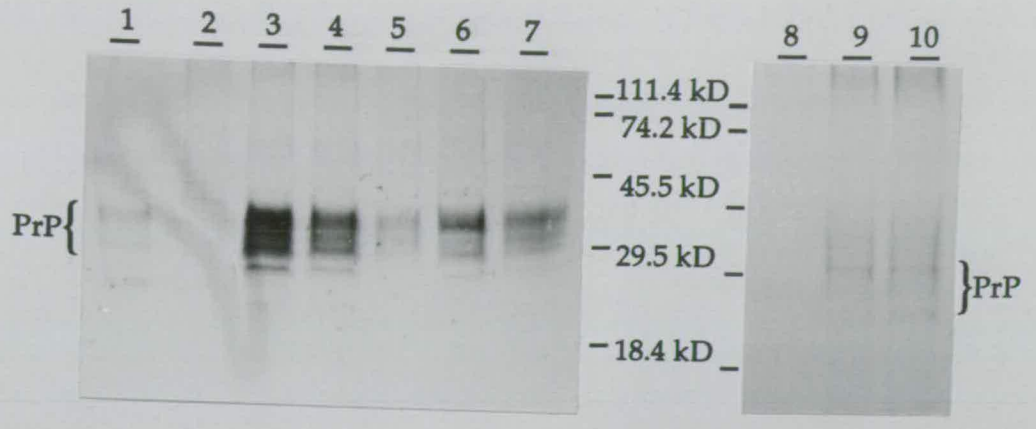


figure 4.6.1

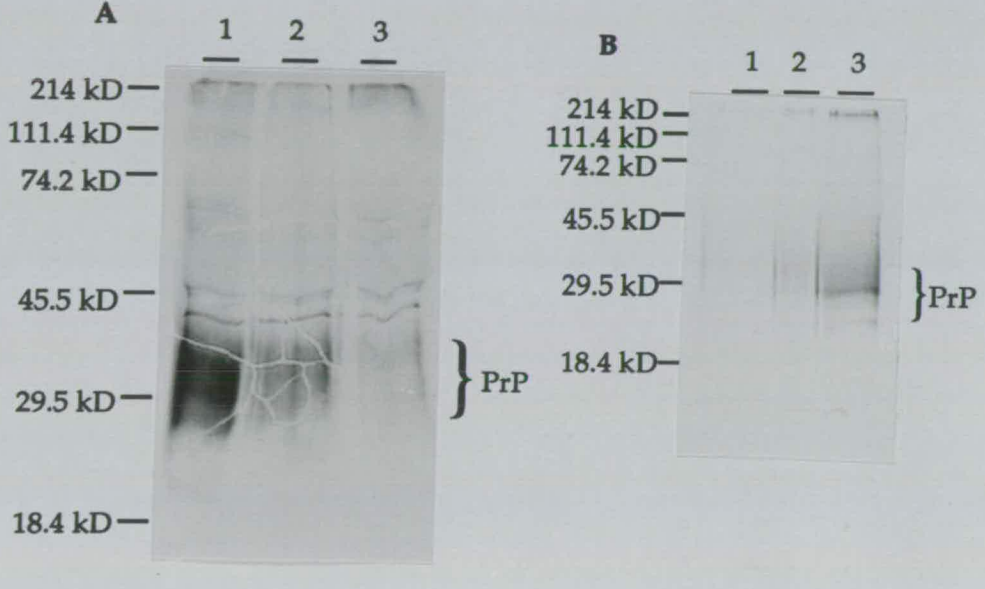


figure 4.6.2

Immunoprecipitations were performed with the monoclonal anti-PrP antibody (ab) 3F4 (Kascsak *et al.*, 1987) usually at 1:500 dilution, or the polyclonal antibodies 1B3, or 1A8 (Farquhar *et al.*, 1989) usually 1:1000 dilution. The 3F4 ab recognises the protein produced only by the constructs (HaPrP) and not the endogenous mouse or monkey PrP of the N2a or cos7 cells respectively. The polyclonal antibodies were used to show the endogenous amounts of PrP and in one experiment they were used for a comparison between the translation of the endogenous message and that produced by the constructs. The ip results could be quantified by phosphorimager. One of the first attempts to compare the amount of HaPrP protein produced by the different constructs is shown in Figure 4.6.1. Precise conclusions could not be made because there were no data on the amount of HaPrP mRNA that these transfected lines produced (there were insufficient cells for RNA extraction). However, these results suggested that there was a difference in the translation efficiency and the processing of HaPrP between N2a and cos7 cells.

To investigate whether the turnover of PrP differs between cos7 and N2a cells, the half-life of the HaPrP protein produced by one of the constructs (pC HaEPU) was determined. The protein extract from both transfected cell lines was immunoprecipitated with 3F4 ab after 4 and 8 hours chase period following a 2 hour pulse period (section 7.22.2). The results of that experiment is shown in Figure 4.6.2. These data suggested that there is no major difference in HaPrP turnover between the two cell lines.

The comparison between one of the N2a stable lines (Figure 4.6.1, lane 5) and the transiently transfected lines (Figure 4.6.1, lanes 3, 4, 6 and 7) revealed that HaPrP protein levels in the transiently transfected cells were much higher, probably due to the higher amounts of mRNA achieved by this method (high transfection efficiency). In the following experiments, accurate quantification of the translation efficiencies between the different HaPrP constructs was attempted.

Transient transfections were performed in parallel for both N2a and cos7 cells and control transfections were done with the pCEP4 vector. One portion of the transfected cells was kept for RNA analysis and the remainder was ^{35}S-labeled and the protein extract immunoprecipitated. The Northern blots were hybridised with a HaPrP ORF-containing fragment (HaP 940 bp, see Figure 4.2.1) and the result

quantified by phosphorimager. In order to make the quantifications more precise, Northern blots were stripped and re-hybridised with a probe containing sequences from the mouse ribosomal protein S26. Values obtained from the phosphorimager were first expressed in relation to values obtained from the control transfection (N2a, or cos7 cells transfected with pCEP4 construct). The values obtained from the hybridisation with HaP were normalised to control hybridisation (S26) values. Because cos7 are monkey cells, hybridisation with mouse S26 would not produce as strong a signal as for N2a cells and therefore all comparisons between cos7 and N2a cells were done relying on ethidium bromide staining of the RNA gels.

Before performing immunoprecipitations with the monoclonal 3F4 ab or the polyclonal 1B3 (or 1A8) ab, aliquots of the protein extracts were kept for TCA precipitations (section 7.24). TCA precipitation values from cells transfected with the different constructs were expressed in relation to the value obtained from the control transfection. The quantification of HaPrP protein was done by phoshorimager analysis. The values obtained were first expressed in relation to the value obtained from control-transfected cells, and then normalised against the TCA precipitation values. The ratio of the final values from the immunoprecipitations to the final mRNA values would give the translation efficiency for each construct.

Figure 4.6.4A shows a Northern blot of samples from cos7 and N2a cells transfected with several constructs, hybridised with HaP and S26. Immunoprecipitation of HaPrP protein produced by these lines is shown in Figure 4.6.3. After all calculations were made (Figure 4.6.5A) the translation efficiency of the constructs is shown as a percentage and as an histogram (Figure 4.6.5B). This experiment confirmed previous observations on the differences in PrP translation between N2a and cos7 cells and the levels of HaPrP mRNA and protein between the stable and the transient cell lines. The comparison of translation efficiency between the constructs indicates that there are no major differences. This experiment shows that including quantification of the mRNA is crucial for the estimation of translation efficiency; major differences between protein levels produced from each construct might reflect only the differences in the levels of mRNA. Differences in the amount of mRNA might be due to different transcription efficiencies or different

Figure 4.6.3

Immunoprecipitation of transfected cell lines with the 3F4 mouse monoclonal Ha PrP specific ab.

Northern blot analysis of the transfected cell lines presented here is shown in Figure 4.6.4A.

	cells	transfection	construct
1.	cos7	transient	pE HaKo
2.	cos7	transient	pE HaEPU
3.	cos7	transient	pE HaP
4.	cos7	stable	pC HaPU
5.	cos7	stable	pC HaP
6.	cos7	transient	pCEP
7.	cos7	transient	pC HaKo
8.	cos7	transient	pC HaEPU
9.	N2a	transient	pE HaKo
10.	N2a	transient	pC HaKo
11.	N2a	transient	pE HaP
12.	N2a	transient	pE HaEPU
13.	N2a	stable	pC HaEPU
14.	N2a	stable	pC HaPU
15.	N2a	stable	pC HaP
16.	N2a	stable	pCEP

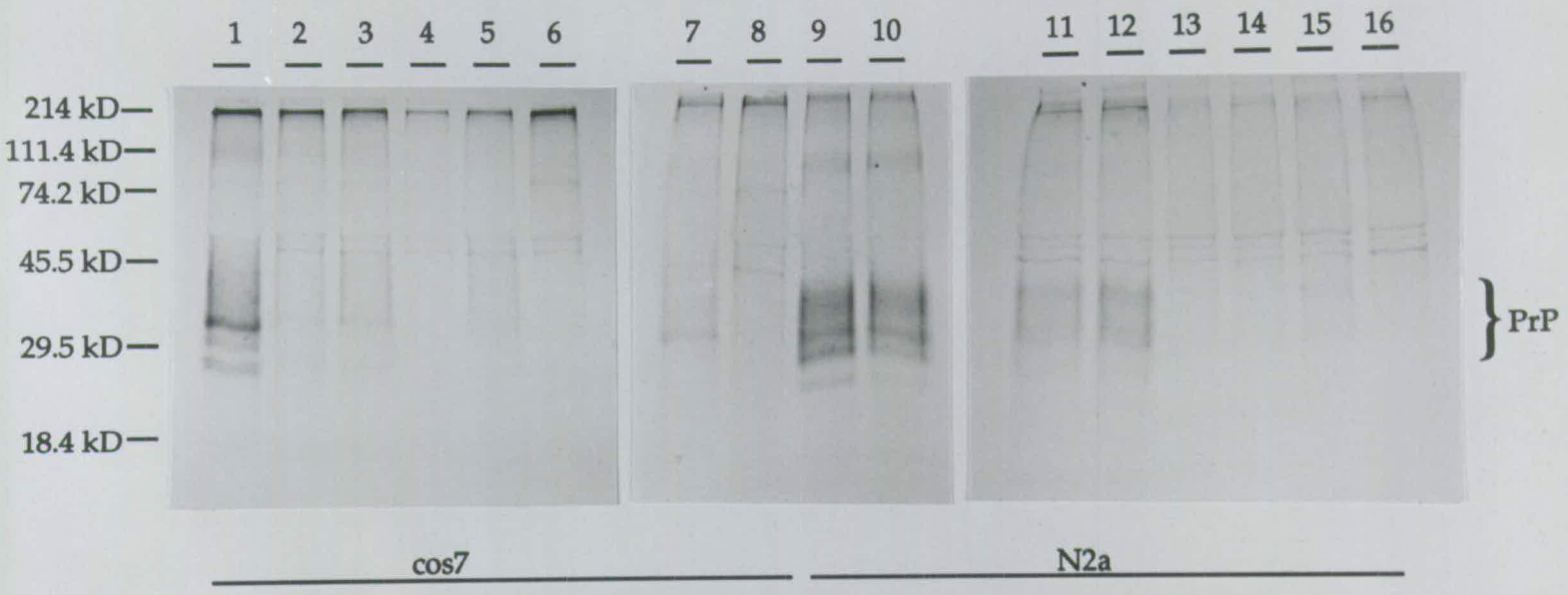

1 2 3 4 5 6
7 8 9 10
11 12 13 14 15 16
214 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
cos7
N2a

figure 4.6.3

Figure 4.6.4

A. Northern blot analysis of transfected cell lines

The probe used was the HaP fragment (940 bp, Figure 4.2.1)

	cells	transfection	construct
1.	N2a	transient	pCEP
2.	N2a	transient	pE HaP
3.	N2a	transient	pE HaEPU
4.	N2a	transient	pE HaKo
5.	N2a	transient	pC HaKo
6.	N2a	transient	pC HaKo
7.	N2a	stable	pC HaP
8.	N2a	stable	pC HaPU
9.	N2a	stable	pC HaEPU
10.	cos7	transient	pCEP
11.	cos7	transient	pE HaP
12.	cos7	transient	pE HaEPU
13.	cos7	transient	pE HaEPU
14.	cos7	transient	pE HaKo
15.	cos7	transient	pC HaKo
16.	cos7	transient	pC HaKo
17.	cos7	transient	pC HaEPU

Samples 5, 12 and 15 are from a previous experiment and do not correspond to any of the cell lines analysed by ip shown in Figure 4.6.3.

B. Northern blot analysis transiently transfected cell lines

The probe used was the HaPrP fragment (940 bp, Figure 4.2.1)

	cells	construct
1.	mouse brain	
2.	mouse brain 1:2 dilution	
3.	N2a	pCEP
4.	N2a	pC HaP
5.	N2a	pC HaPU
6.	N2a	pC HaEPU
7.	N2a	pC HaKo
8.	N2a	pE HaKo
9.	N2a	pC HaPd5'

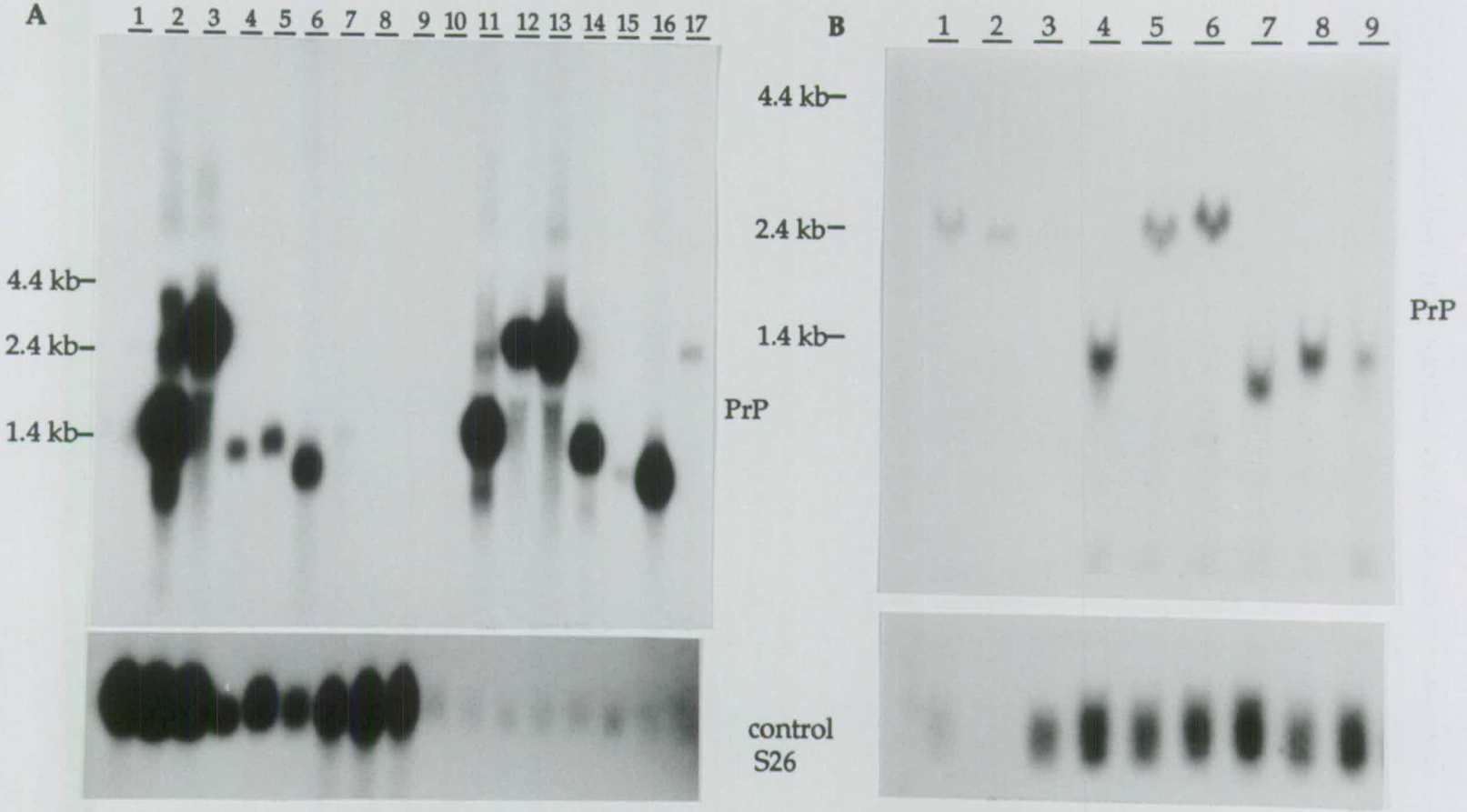

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
4.4 kb–
2.4 kb–
1.4 kb–
PrP
control
S26
B
1 2 3 4 5 6 7 8 9
4.4 kb–
2.4 kb–
1.4 kb–
PrP

figure 4.6.4

Figure 4.6.5

In **A** the values shown in each column were expressed in relation to values obtained from the cos7 cells transfected with the pC HaEPU construct (the lowest ip value, line 9). The ip values (column a, based on Figure 4.6.3) were normalised against TCA precipitation (b) as shown in column c. The mRNA values obtained by hybridisation with the HaPrP fragment (column d, based on Figure 4.6.4A) were normalised against the control hybridisation values with S26 (column e, based on Figure 4.6.4A) as shown in column f. Finally the ratio of normalised ip values (c) to normalised mRNA values (f) gave the translation efficiency values (column g, c/f) shown also as a percentage (column h, g%) and as histogram **B** (g%).

A

	column	a	b	c	d	e	f	g	h
cells	construct	ip	TCA	a/b	mRNA	con.	d/e	c/f	g%
N2a	1 pE HaP	3.1	0.28	11.07	64	37.6	1.70	6.50	70%
N2a	2 pE HaEPU	2.28	0.18	12.67	47	27	1.74	7.28	80%
N2a	3 pE HaKo	5.4	0.46	11.74	4	3	1.33	8.80	100%
N2a	4 pC HaKo	3.6	0.37	9.73	6.24	3.75	1.66	5.85	66%
cos7	5 pE HaP	1.3	0.25	5.20	17.2	6.6	2.61	2.00	23%
cos7	6 pE HaEPU	1.3	0.25	5.20	18.4	6.3	2.92	1.78	20%
cos7	7 pE HaKo	4.7	1.02	4.61	5.2	3	1.73	2.66	30%
cos7	8 pC HaKo	1.3	0.37	3.51	7.2	3	2.40	1.46	16%
cos7	9 pC HaEPU	1	1	1	1	1	1	1	12%

B

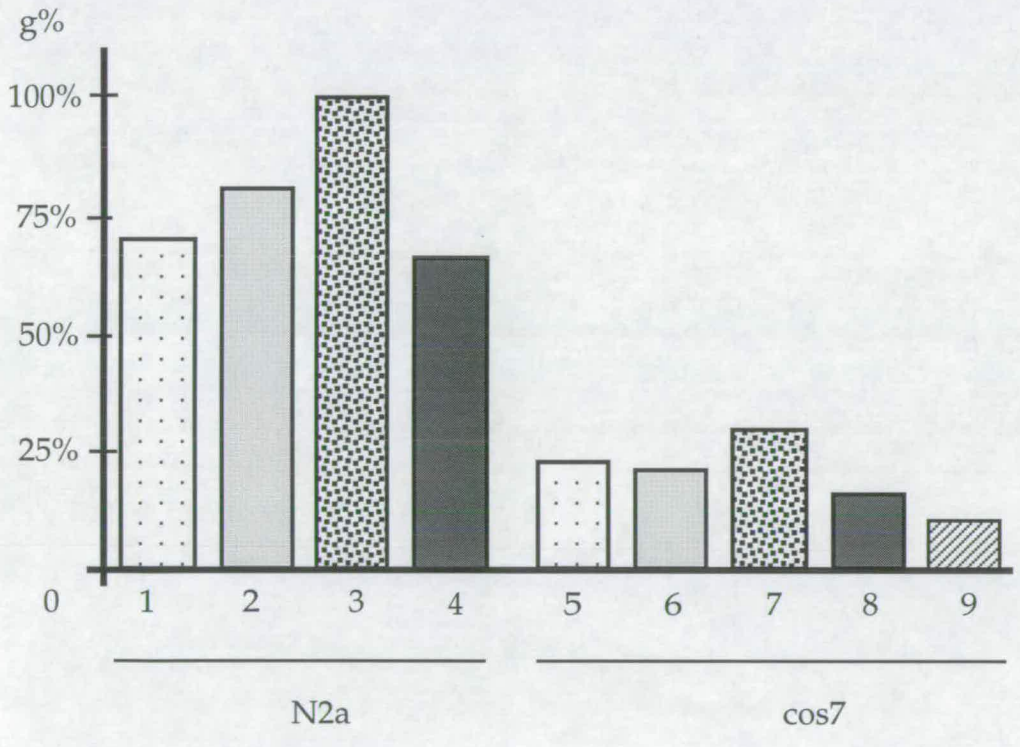


Figure 4.6.5

transfection efficiencies, but since that was not the issue here, it was not further pursued.

In order to make the quantifications more accurate and control for possible mistakes in the immunoprecipitation procedure (loss of part of the protein extract or of the protein-A-sepharose beads, uneven loading, etc.) 1/10 aliquot of the protein extract was immunoprecipitated separately and two loadings (4/5 and 1/5) of the two final immunoprecipitated protein extracts were performed. Transfections were done in N2a cells. The constructs included in this comparison were pC HaP, pC HaPU, pC HaEPU pC HaKo and pC HaPd5'. Of particular importance for this experiment was the use of pC HaPd5' construct, which is the equivalent of the construct used for the generation of transgenic mice (N-terminal truncation of the HaPrP ORF). The Northern blot analysis that corresponds to this experiment is shown in Figure 4.6.4B and the immunoprecipitation results in 4.6.6. Calculations are not shown, but were done as presented in Figure 4.6.5 and the comparison in translation efficiencies is presented as a percentage and as a histogram in Figure 4.6.7. Four quantifications correspond to each transfected cell line because each protein extract was split in two samples that were immunoprecipitated, and two loadings were performed for each sample. The average of all four and the average of two (corresponding to ip1 and ip2, Figure 4.6.6) of the quantifications was estimated and the translation efficiencies based on these estimations are presented in column a, and column b respectively, in Figure 4.6.7. In this way any major deviations due to mistakes regarding one of the four gels should become obvious.

These data demonstrated again that there were no major differences in translation efficiency between the different constructs apart from pC HaPd5', where the second ATG of HaPrP was used as the initiating codon (the first 32 nucleotides were deleted).

Comparison of the translation efficiency between a few of the constructs was repeated. Also, in order to do a comparison with the endogenous MoPrP protein levels, both monoclonal and polyclonal antibodies were used. In addition the pC HaPd5' construct (truncated HaORF) was tested again. The Northern blot analysis of this experiment is shown in Figure 4.6.8A and the two protein gels with

Figure 4.6.6

Immunoprecipitation of transiently transfected cell lines with the 3F4 mouse monoclonal HaPrP specific ab.

The samples on each gel are in the same order.

The RNA analysis of the transfected cell lines presented here is shown in Figure 4.6.4B.

1/10 of the the protein extract that was immunopreciopitated and electrophorised on gel A was immunoprecipitated and electrophorised on gel B. Before loading 1/5 of the samples shown in A were loaded on gel C and 1/5 of the samples shown in B were loaded on gel D.

	cells	construct
1.	N2a	pCEP
2.	N2a	pC HaP
3.	N2a	pC HaPU
4.	N2a	pC HaEPU
5.	N2a	pE HaKo
6.	N2a	pC HaKo
7.	N2a	pC HaPd5'

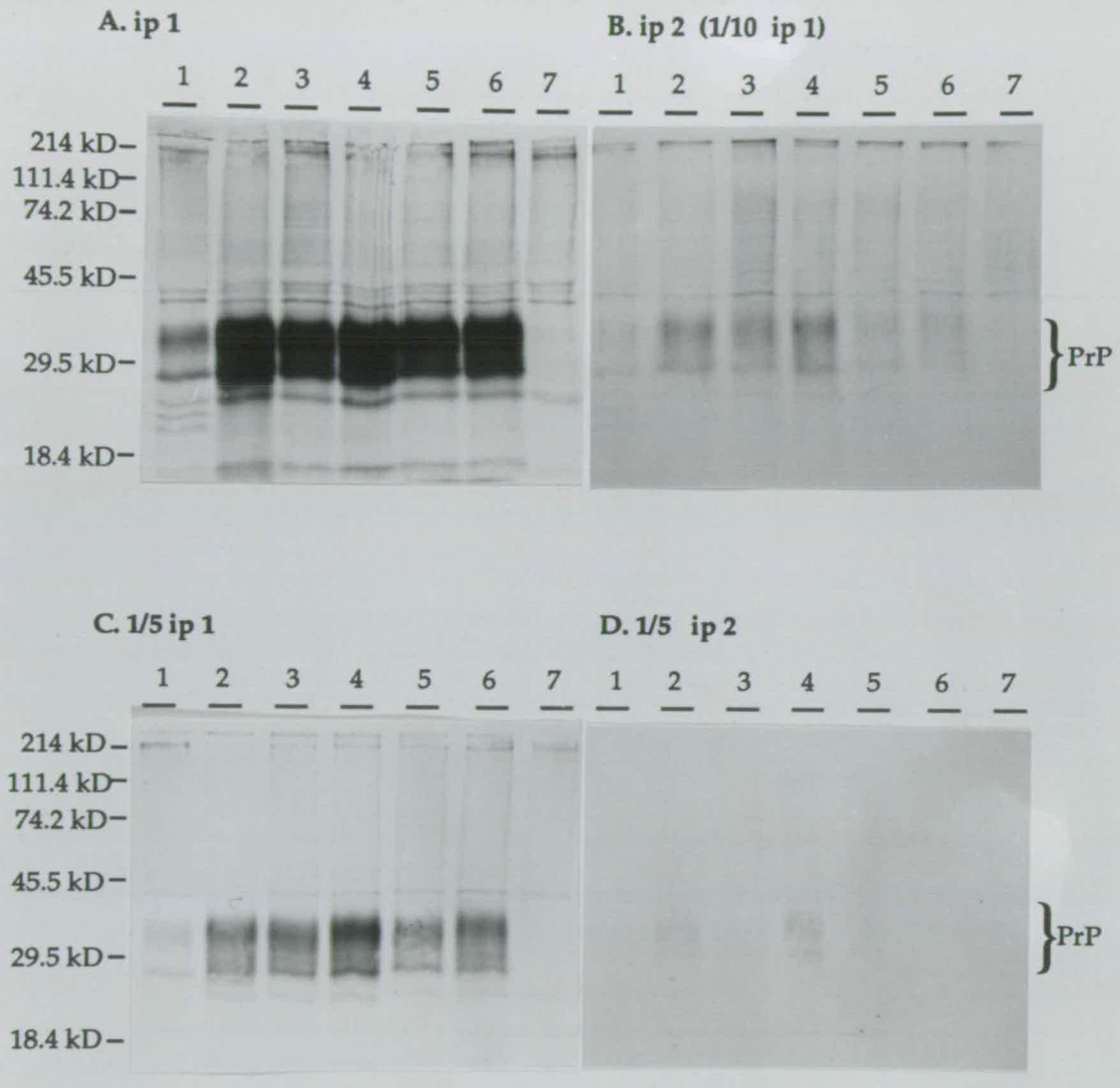

A. ip 1
B. ip 2 (1/10 ip 1)
1 2 3 4 5 6 7
214 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C. 1/5 ip 1
D. 1/5 ip 2

figure 4.6.6

cells		construct	a	b	a%	b%
N2a	1	pCEP				
N2a	2	pC HaP	22.36	33.7	86%	87%
N2a	3	pC HaPU	21.61	30.0	83%	78%
N2a	4	pC HaEPU	25.72	42.8	100%	100%
N2a	5	pE HaKo	20.78	36.0	80%	99%
N2a	6	pC HaKo	16.27	31.65	65%	83%
N2a	7	pC HaPd5'	1	1	0.5%	0.4%

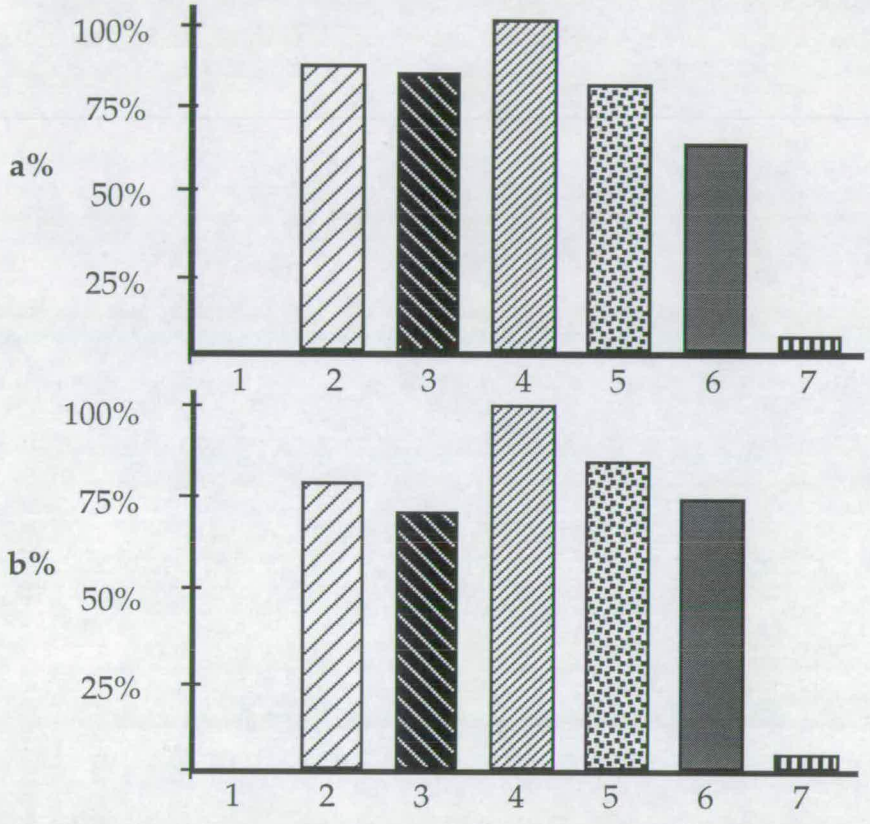


Figure 4.6.7

All values in columns a and b are expressed in relation to values obtained from transfection with pCHaPd5' construct.

Column **a** is showing the values corresponding to the translation efficiency of each construct when all four ip gels (figure 4.6.6) were included in the calculations.

Column **b** is showing the values corresponding to the translation efficiency of each construct when ip1 and ip 2 (gels A and B) were included in the calculations. **a** and **b** are also presented a percentage (**a%** and **b%**) and as a histogram.

Figure 4.6.8

A. Northern blot analysis transiently transfected cell lines

The probe used was the HaP fragment (940 bp, see Figure 4.2.1)

1. mouse brain

	cells	construct
2.	cos7	pCEP
3.	cos7	pC HaEPU
4.	N2a	pC HaEPU
5.	N2a	pC HaEPU
6.	N2a	pE HaKo
7.	N2a	pE HaKo
8.	N2a	pC HaPd5'
9.	N2a	pC HaPd5'

Samples 4, 7 and 9 are from a previous experiment and do not correspond to any of the cell lines analysed by ip shown in B and C.

B. and **C.** Immunoprecipitation of transiently transfected cell lines with the 3F4 mouse monoclonal Ha PrP specific ab and the 1A8 rabbit polyclonal Anti-PrP ab.

	cells	construct	ip
1.	N2a	pCEP	3F4 ab
2.	N2a	pCEP	1A8 ab
3.	N2a	pC HaEPU	3F4 ab
4.	N2a	pC HaEPU	1A8 ab
5.	N2a	pE HaKo	3F4 ab
6.	N2a	pE HaKo	1A8 ab
7.	N2a	pC HaPd5'	3F4 ab
8.	N2a	pC HaPd5'	1A8 ab
9. -			
10.	cos7	pCEP	1A8 ab
11.	cos7	pC HaEPU	1A8 ab

Gel **C** represents a 1:5 loading dilution of samples shown in **B**. Sample 8 on gel **C** was lost during the loading procedure. Quantifications shown in Figure 4.6.9 are based on gel **B**.

The signal obtained by N2a cells transfected with pCEP construct and immunoprecipitated with the 3F4 ab is due to background hybridisation (lane 1 gels **B, C**).

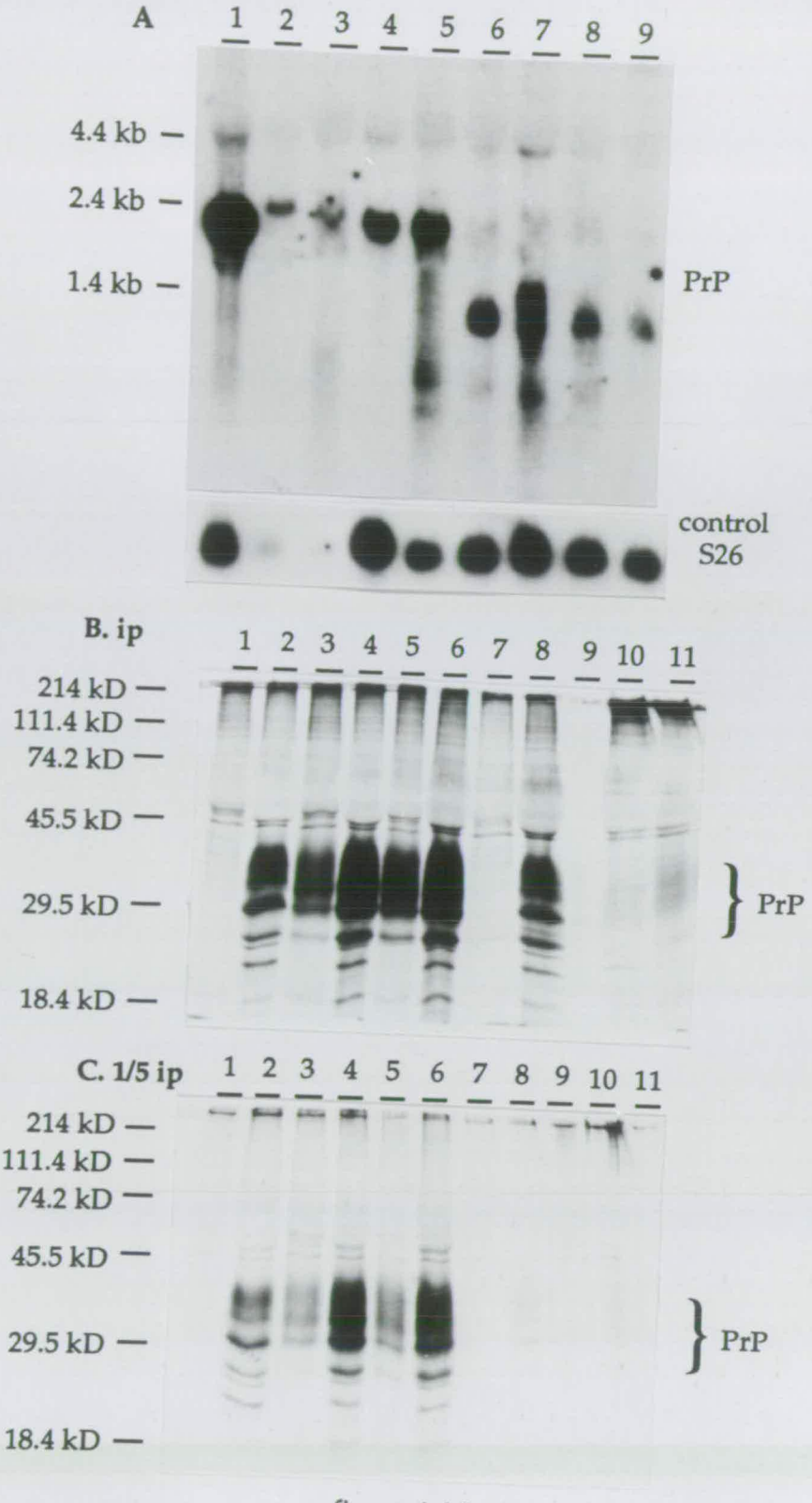

A
1 2 3 4 5 6 7 8 9
4.4 kb
2.4 kb
1.4 kb
PrP
control
S26
B. ip
1 2 3 4 5 6 7 8 9 10 11
214 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C. 1/5 ip
1 2 3 4 5 6 7 8 9 10 11
214 kD
111.4 kD
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 4.6.8

Figure 4.6.9

A. Comparison of translation efficiency between different constructs

All values were expressed in relation to values obtained from N2a cells transfected with pC HaPd5' construct (the lowest ip value).

The ip values in column a, were estimated on the samples immunoprecipitated with the 3F4 ab: lanes 3, 5, 7, gel **B.ip**, Figure 4.6.8. These values were normalised against TCA precipitation (b) as shown in column c. The mRNA values obtained by hybridisation with the HaP fragment (column d, based on **A** Figure 4.6.8) were normalised against the control hybridisation values with S26 (column e, based on **A** Figure 4.6.8) as shown in column f. The ratio of ip values (c) to mRNA values (f) gave the translation efficiency for each construct (column g, c/f).

B. Comparison of translation efficiency between endogenous and transfected HaPrP

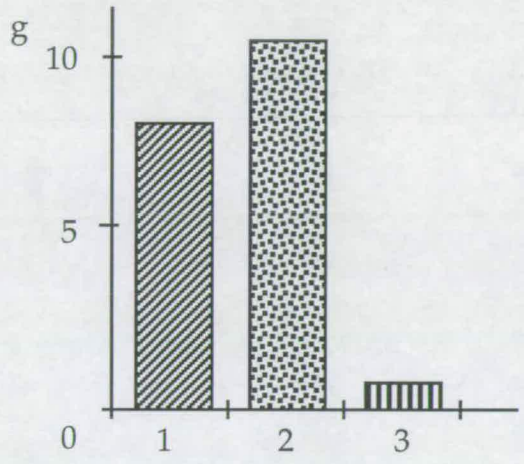
All values were expressed in relation to values obtained from N2a cells transfected with pCEP construct.

The ip' values in column a are the original numbers obtained by phosphorimager analysis based on samples immunoprecipitated with the 1A8 ab: lanes 2, 4, 6, 8, gel **B.ip** (Figure 4.6.8), and represent the signal from both the endogenous MoPrP and the HaPrP produced from the constructs. Therefore the value corresponding to the endogenous MoPrP signal (control pCEP-transfected N2a cells: 134728) was subtracted from the values obtained from the transfected N2a cells as shown in column b (ip''). These values were expressed in relation to the value obtained from control pCEP-transfected N2a cells (column c) and were subsequently normalised against TCA precipitation (column d) as shown in column e. mRNA values (f) were normalised against control hybridisation values (g) as shown in column h. The ratio between normalised ip values (e) were normalised against the normalised mRNA values (h) to give the translation efficiency values (column i).

*The mRNA levels representing the endogenous MoPrP message were estimated on lane 8, Figure 4.6.8A and not on control-transfected N2a.

A

	a	b	c	d	e	f	g
construct	ip	TCA	ip/TCA	RNA	co	d/e	c/f
1 pC HaEPU	13.35	0.79	16.90	1.49	0.72	2.07	8.17
2 pE HaKo	16.81	1.13	14.88	1.24	0.89	1.39	10.68
3 pC HaPd5'	1	1	1	1	1	1	1



B

	a	b	c	d	e	f	g	h	i
construct	ip'	ip''	ip	TCA	ip/TCA	mRNA	co	f/g	e/h
1 pCEP4	134728	134728	1.00	0.81	1.23	0.15 *	1.00*	0.14	8.82
2 pC HaEPU	361582	226854	1.68	0.79	2.13	1.49	0.72	2.07	1.03
3 pE HaKo	377524	242796	1.80	1.13	1.59	1.24	0.89	1.39	1.14
4 pC HaPd5'	135998	1270	0.01	1	0.01	1	1	1.08	0.01

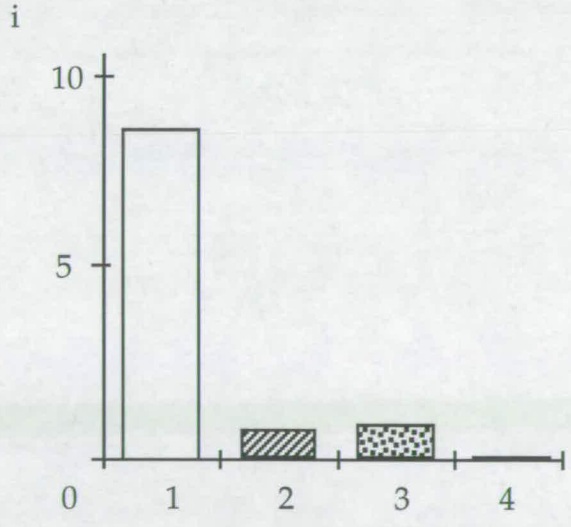


figure 4.6.9

immunoprecipitated samples in Figure 4.6.8B,C. Each protein extract was separated into two equal aliquots; one was immunoprecipitated with the 3F4 ab and the other with the 1A8 ab. Transfection of cos7 cells was also performed, but the low mRNA levels (Figure 4.6.8A, lane 3) suggested that the transfections did not work very efficiently. Therefore, the HaPrP protein levels were very low (Figure 4.6.8B,C lanes 10, 11) and these samples were not included in the calculations. A loading dilution (1:5) was also performed (4.6.8C). Quantification of immunoprecipitations were based on gel B (Figure 4.6.8B) and all values were expressed in relation to those obtained from N2a cells transfected with pCEP4 construct. The calculations are shown in Figure 4.6.9 and the translation results are presented both as a percentage and as a histogram (Figure 4.6.9). These results suggest that there is more endogenous than transgene protein produced and confirm that the pC HaPd5' construct is very inefficiently translated. Furthermore a comparison between transgenic and normal mouse brain, transfected and non-transfected N2a cells was performed. The data in Figure 4.6.10 show that although PrP mRNA levels are very high in transfected N2a cell lines the translation efficiency is much lower than in the brain. Even if detectable amount of PrP protein was produced in the transfected cell lines it could be masked by the high background produced by the 3F4 ab. Low translation efficiency in comparison to brain is observed in all other tissues. The transgene-produced mRNA in Tg mouse brain is translated very inefficiently (if at all) due to the 5' truncation of the HaPrP fragment and therefore not detectable .

4.6.2 Conclusions

The data presented above indicate that there was no significant difference in translation efficiency produced by the addition of the 3' or 5' UTR of HaPrP, although the constructs that include the entire 5' UTR show up to 2-fold better translation efficiency. The presence of intron sequences in the vector does not appear to have any influence on translation efficiency. The most dramatic effect is observed with the pC HaPd5' construct where the first 32 nucleotides were deleted. This construct appears to show barely any protein translation (approximately

3%). When the initiation of translation sequence of this HaPrP fragment is compared to the consensus sequence and to that surrounding the first ATG of HaPrP, it does not appear to be similar (Figure 4.6.11). Especially the nucleotide at the crucial position +4 (regarding initiation of translation), which is T instead of G, has been shown to be responsible for five to ten-fold downregulation of translation (Kozak, 1987a). The nucleotide at position -3 (another crucial position), is a G instead of A, which is acceptable. The translation of this protein is therefore expected to be very low and furthermore, because it is missing the signal peptide, possibly it is not processed properly and might be degraded very quickly. The very small amount detected here (Figure 4.6.6, lane 7 and Figure 4.6.8B, lane 7) is almost equivalent to background levels.

Comparison of translation efficiencies in transfected and non-transfected N2a cells suggested that the endogenous message is translated better than that produced by the transgene. Both endogenous and transgene-produced PrP mRNA are translated better in neuronal cell lines and in the brain.

Figure 4.6.10

A. Northern blot analysis cell lines and mouse tissues

The probe used was the HaPrP fragment (940 bp, see Figure 4.2.1)

	cells	transfection	construct
1.	N2a	transient	pC HaKo
2.	N2a	transient	pCEP

3. Tg NSE 14 brain
4. F1 mouse brain
5. Tg NSE 20 brain
6. Tg NSE 20 heart
7. Tg NSE 20 lung
8. Tg NSE 20 liver
9. Tg NSE 20 spleen
10. Tg NSE 20 kidney

B. and **C**. Western blot analysis of cell lines and mouse tissues

B. Probed with the mouse monoclonal HaPrP-specific ab 3F4

C. Probed with the rabbit polyclonal anti-PrP ab 1A8

The samples on both blots are in the same order

1. mouse brain
2. Tg NSE 14 brain
3. Tg NSE 20 brain
4. Tg NSE 20 heart
5. Tg NSE 20 lung
6. Tg NSE 20 liver
7. Tg NSE 20 spleen
8. Tg NSE 20 kidney

9.	N2a	transient	pCEP
10.	N2a	transient	pC HaP
11.	N2a	transient	pC HaPU
12.	N2a	transient	pC HaKo

No RNA data are shown for transiently transfected N2a with pC HaP and pC HaPU construct (lanes 10 and 11).

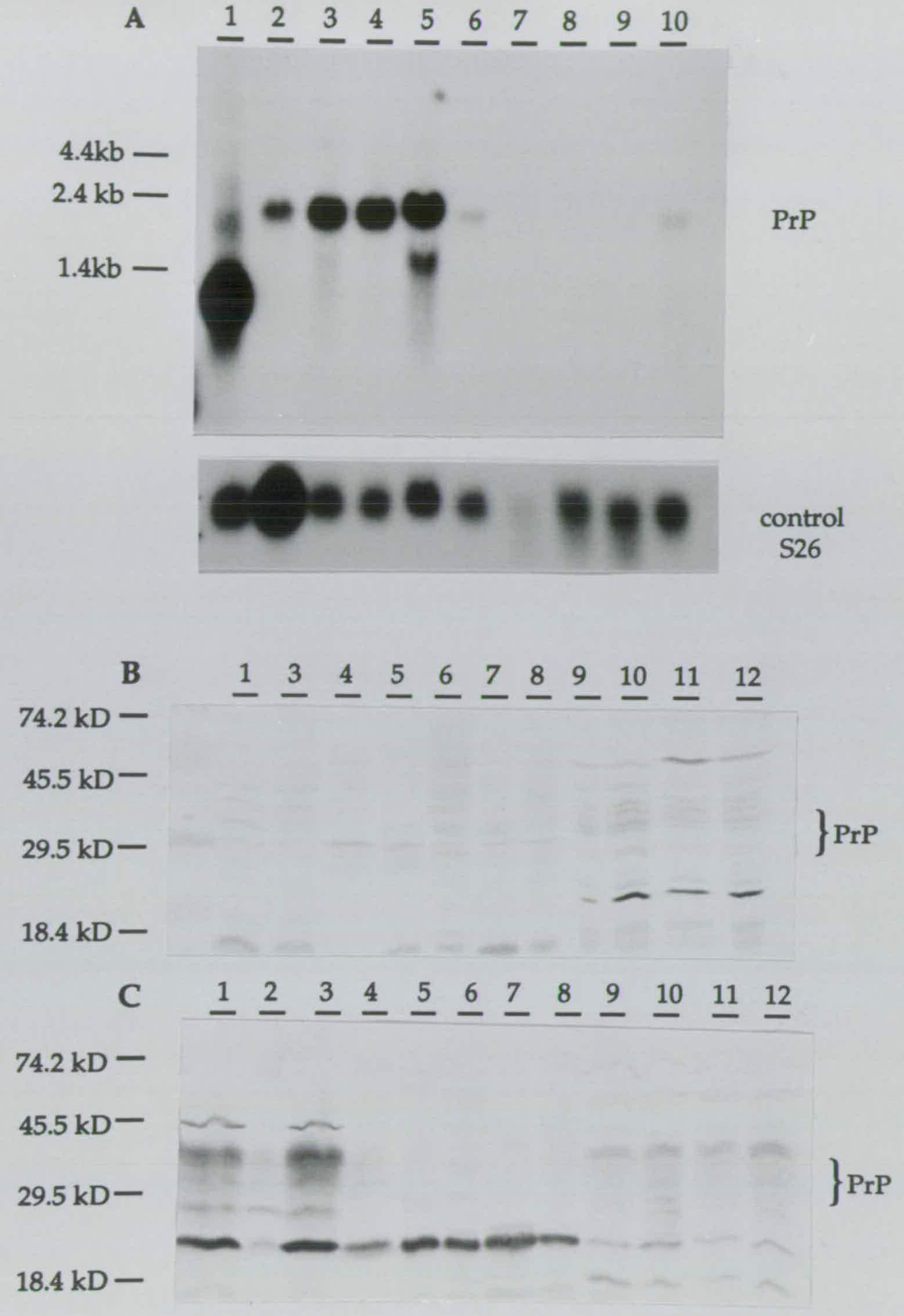

A
1 2 3 4 5 6 7 8 9 10
4.4kb
2.4 kb
1.4kb
PrP
control
S26
B
1 3 4 5 6 7 8 9 10 11 12
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP
C
1 2 3 4 5 6 7 8 9 10 11 12
74.2 kD
45.5 kD
29.5 kD
18.4 kD
PrP

figure 4.6.10

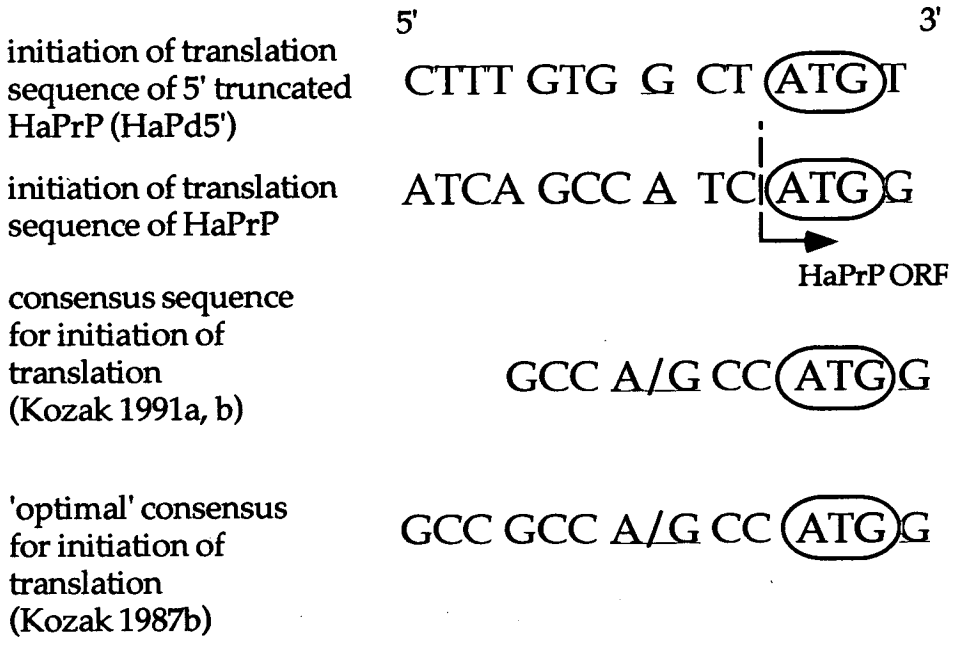


Figure 4.6.11 Comparison of the initiation of translation sequence for the different HaPrP fragments

CHAPTER 5

General discussion

In this chapter the conclusions and the work discussed in previous chapters will be summarised. The objective of the project was to study the role of PrP in the etiology of TSEs by exploiting neuron-specific and region-specific expression of heterologous PrP in the brains of transgenic mice (chapter 2). The effect that this would have on the susceptibility, pathogenesis and species barrier of the disease was to be investigated. Also the ability of the isolated HaPrP ORF to produce HaPrP protein was assessed. However, problems were encountered with PrP protein translation in the transgenic mice generated and the original objectives (see also section 1.14) could not be met. Although trivial reasons account for the failure to express HaPrP protein in the transgenic mice generated, accumulating data from other studies suggested that sequences flanking the PrP ORF might contain regulatory sequences (section 2.8). Therefore, possible effects of the 5' and 3' untranslated sequences of HaPrP on the translation of lacZ and HaPrP were investigated (chapters 3 and 4).

Neuron-specific expression of HaPrP in transgenic mice was attempted by using 5' regulatory sequences of the NSE gene. The levels of transgene mRNA in the Tg NSE HaPrP mice were low in comparison to endogenous PrP levels (section 2.8). Recent experiments have demonstrated that the presence of intron 1 together with the 5' upstream sequences are necessary for enhanced neuron-specific expression in cell culture (Sakimura *et al.*, 1995). Furthermore, in a recent study a larger fragment of NSE 5' flanking sequences, together with exon 1, intron 1 and a very small portion of exon 2 was used to drive the expression of HaPrP in transgenic mice (Race *et al.*, 1995). The amount of transgene RNA in the single transgenic line generated was 5-fold higher than the endogenous MoPrP. These data indicate that regulatory elements necessary to produce high level expression were probably missing from the 1.8 kb fragment of the 5' flanking sequence of the NSE gene used here. However, the possibility of variegated expression of the transgene observed in other transgenic models (Festenstein *et al.*, 1996; Dobie *et al.*, 1996), could also explain the low levels of transgene expression. The

transgene could be expressed at high levels in some neurons, while it could be silenced in others and therefore, when total brain samples were analysed only low levels of PrP mRNA could be detected.

The amount of HaPrP mRNA in the Tg NSE 20 line should have been sufficient to generate detectable HaPrP protein. The use of a 5' truncated HaPrP cDNA (Oesch *et al.*, 1985) was thought to have an effect on the translation efficiency of the transgene mRNA and/or the processing of the protein. This effect was investigated in cell culture by comparing the translation efficiency of the 5' truncated HaPrP fragment with other HaPrP fragments (discussed in more detail below).

A general point emerging from quantitative analysis of PrP mRNA and protein expression when tissues other than brain, were included in the analysis, is that translation of PrP appears to be more efficient in the brain, which agrees with quantitative analyses made recently by other groups (Horiuchi *et al.*, 1995). If PrP mRNA was translated as efficiently as that in the brain, PrP protein should be detected by the method used here at least in some other tissues apart from brain, including heart and lung (PrP mRNA is approximately 25% in comparison to that detected in the brain). However, no PrP protein was detected in any tissue other than brain. In agreement with these data were the conclusions from translation experiments in cell culture.

Both endogenous and transgene-produced PrP mRNA are translated better in neuronal cell lines. Non-neuronal cell lines are also capable of producing PrP, but the translation efficiency is much lower and there could be differences in the processing of the protein. Although detection of PrP protein by Western blot analysis was performed successfully in brain protein extracts, difficulties were sometimes encountered with protein extracts from cultured cells. This suggested that the brain shows the highest levels of PrP translation and therefore PrP protein detection is much easier in that tissue regardless the detection method applied. Another finding was that the endogenous PrP message is translated more efficiently than the transgene-produced PrP in cultured cells. This could be because the endogenous message is either more stable or translated more efficiently. The turnover of the endogenous protein may also be slower, or the capacity of the cells to produce larger amounts of PrP than normal might be limited. The low

levels of transgene-produced mRNA probably made the detection of PrP protein more difficult.

Comparison between constructs containing different fragments of HaPrP cDNA demonstrate that the addition of the 3' or 5' UTR of HaPrP does not have a major effect on the translation efficiency of either lacZ or PrP. Constructs that include the entire 5' UTR show up to 2-fold increase in translation efficiency, and a slight downregulation (to 79-90%) in translation efficiency was observed with constructs that include the entire 3' UTR (especially with the lacZ constructs, see tables 3.4.1,2 chapter 3). These differences were not considered statistically significant.

Most constructs used previously in cell culture contained only the PrP ORF, but a larger fragment of HaPrP cDNA (1.3 kb), including the 5' UTR, the HaPrP ORF and a part of the 3' UTR (~500 bp) has also been used (Chesebro *et al.*, 1993). When expressed in murine neuroblastoma cells a 60 kD protein containing HaPrP was identified in addition to the ~25-40 kD species (Kocisko *et al.*, 1994; Priola *et al.*, 1995). It was suggested that this protein was a PrP dimer that was sensitive to proteinase K digestion. The dimer was not dissociated by denaturing treatments and tended to form large aggregates. Such forms have been identified in scrapie infected hamster brain, but the cells (MNB: mouse neuroblastoma) where the 60 kD PrP was identified were not infected with the disease agent. It is not clear whether the presence of the 60 kD protein was due to the use of a certain construct, the overexpression of HaPrP, improper processing or folding of HaPrP, a specific step during the isolation or immunoprecipitation of HaPrP, or the specific type of cells. The only PrP species identified in both cos7 and N2a cells transfected with any of the constructs used here were the ~25-40 kD species.

The use of intron sequences in the vector does not appear to have any influence on translation efficiency. The only construct that showed extremely low translation efficiency was the one containing the same HaPrP fragment as that used in the transgenic construct (the first 32 nucleotides were deleted). The 'weak' consensus sequence surrounding the translation initiation codon appeared probably to be responsible for downregulation of translation. Even if some HaPrP protein was produced, the deletion of part of the signal peptide would affect further

processing of the protein. This result explains the absence of detectable amount of protein produced in the Tg NSE HaPrP transgenic mice.

Recently transgenic mice have been generated containing 1 kb of the HaPrP sequence (including the 5' UTR, the HaPrP ORF and a small part of the 3' UTR) (Race *et al.*, 1995). PrP expression was driven by the NSE promoter (containing NSE intron I, also see above and 1.12.1) that gave high and neuron-specific production of HaPrP protein. These mice were shown to be susceptible to the hamster infectious agent and developed degeneration of the brain. These data indicated that the intron and the 3' UTR are not necessary for PrP protein expression, in agreement with the translation experiments performed in tissue culture (chapter 4). Furthermore, it was shown that PrP sequences not included in the transgene, and sequences flanking the PrP gene included in the cosmid clones previously used, are not necessary for confering susceptibility to the disease. The absence of HaPrP expression in astrocytes, splenic cells and lymph nodes did not restrict replication of the infectious agent in the brain after intracerebral inoculation. However, the involvement of these cells in interspecies transmission following peripheral routes of inoculation was not investigated.

We also attempted to generate transgenic mice that would express PrP in restricted regions of the brain, by using the TH promoter to drive HaPrP expression. The Tg TH HaPrP mice showed very low level of HaPrP mRNA only in some catecholaminergic neurons, indicating that the promoter fragment was possibly missing elements necessary for high-level and specific expression of the transgene. For this reason and because it became apparent that there was a translation problem created by the HaPrP fragment used in the transgenic constructs (see also sections 2.7, 2.8 and comments above), their analysis was not further pursued. Expression of the transgene in some catecholaminergic regions (eg. olfactory bulb) but not in other (eg. substantia nigra) could be due to position effects (section 2.1).

In a recent study (Brandner *et al.*, 1996) a different approach was taken in order to achieve region-specific expression of PrP. Embryonic telencephalic tissue from transgenic mice overexpressing PrP was grafted into the caudo-putamen or the lateral ventricles of Prn-$p^{0/0}$ mice. When these mice were inoculated with the scrapie agent neuropathological changes were detected only in the graft. PrP^{Sc} was

spread by diffusion within the extracellular space to regions surrounding the graft but no pathological changes were observed in these regions. Therefore the infectious agent can traverse unsusceptible brain leaving it unaffected and it can cause degeneration only in neurons that express PrP^{C}. The evidence from these recent studies suggest that PrP^{Sc} can be toxic only when it is produced within the cell, but not when it is introduced from an extracellular source. Another explanation could be that PrP^{C} is needed for the initiation of the conversion of PrP^{C} to PrP^{Sc}, or for the internalisation of PrP^{Sc}.

CHAPTER 6

Materials and methods

Unless otherwise stated, all chemicals were of analytical grade and supplied by BDH Laboratory Supplies or Sigma. Phenol saturated in Tris buffer was supplied by Fisons Bioscience (P/2318/05). Synthetic oligonucleotides were supplied by Oswel. Ethanol was supplied by Hayman. Kodak XAR-5 X-ray film was supplied by H.A. West. Radiochemicals were supplied by Amersham, DuPont-NEN and ICN.

Computer software packages used for DNA and protein analyses were the DNAstar computer package and the University of Wisconsin Genetics Computer Group, (1991), GCG package version 7 (UWGCG).

Computer software packages used for phosphorimager analysis were Molecular Dynamics.

Many of the methods described in this work are based on those described by Sambrook *et al.*, 1989, or in the Promega 'protocols and applications guide'.

7.1 Standard solutions

EDTA
(ethylenediaminetetraacetic acid, disodium salt)
0.5 M Na_2EDTA pH 8.0

TE
10 mM Tris.HCl pH 7.4
1 mM EDTA

20 X TBE
216 g Tris base
110 g boric acid
40 ml 0.5 M EDTA
water to a litre

20 X SSC
3 M NaCl
0.3 M Na_3citrate
pH adjusted to 7.0 with NaOH

1 X PBS (phosphate buffered saline)
0.01 M sodium and potassium phosphate buffer:
0.8% (w/v) NaCl
0.02% (w/v) KCl
0.144% (w/v) Na_2HPO_4
0.024% (w/v) KH_2PO_4
pH adjusted to 7.4 with HCl
or, phosphate buffered saline tablets (SIGMA P-4417) were used

50 X Denhardt's solution
5 g ficoll (Type 400, Pharmacia)
5 g polyvinylpyrrolidone
5 g bovine serum albumin (BSA, Boehringer Mannheim 711454)
water to 500 ml

7.2 Preparation of electrocompetent E. coli

LB growth medium
10 g Bacto-tryptone (Difco 0123-01-1)
5 g Bacto-yeast extract (Difco 0127-01-7)
10 g NaCl
water to 1 l
adjust pH to 7.0 with NaOH and autoclave

E. coli strain XL1 Blue (Stratagene 200130) was used to make electroporation competent cells according to the following protocol. The competence of the resultant cells was usually around 10^8 transformants/µg of plasmid DNA. The E. coli strain JM110 was also used (Stratagene 200239) when NruI restriction enzyme (sensitive to dam methylation) was required for cloning steps.

1. 1 l of LB growth medium was inoculated with a single colony of E. coli and grown in the incubator shaker at 37°C until an OD 600 of 0.6-0.9.

2. The flask was chilled on ice for 5 min and was not allowed to warm up thereafter.

3. The cells were spun in 250 ml bottles (Sorvall) at 4,000 rpm, 4°C for 20 min. The supernatant was removed immediately after centrifugation to prevent lifting of the pellet.

4. Each pellet was gently resuspended in 250 ml of ice cold water, transferred to two pre-chilled 250 ml bottles and spun at 5,000 rpm, 4°C for 10 min. The supernatant was removed, resuspended in 200 ml of ice cold water and spun as before.

5. Each pellet was gently resuspended in 40 ml of ice cold 10% glycerol (BDH) in water, transferred to pre-chilled 50 ml conical tubes (Corning) and spun at 4,000 rpm, 4°C for 10 min.

6. The pellet was resuspended in 2 ml of ice cold 10% (v/v) glycerol per litre of original culture volume, 50 µl aliquots were transferred to pre-chilled microfuge tubes and snap frozen in liquid nitrogen. Store at -70°C.

7.3 Electroporation of E. coli

This method is based on that described by Dower *et al.*, 1988.

SOB

2% (w/v)	Bactotryptone
0.5% (w/v)	Bacto-yeast extract
10 mM	NaCl
2.5 mM	KCl

autoclave, cool, add 1/100 volume 2 M $MgCl_2$-$MgSO_4$

SOC

SOB + 1/100 volume 2M glucose (2 mM final)

LB agar plates

10 g	Bactotryptone

5 g Bacto-yeast extract
10 g NaCl
20 g Bactoagar
make up to 1 l, pH 7.0 with NaOH, autoclave and pour onto 9 cm plates (Corning)

LB agar/X-gal plates
The following solution was spread on 9 cm LB agar plates:
40 µl of 20 mg/ml X-gal (5-bromo-4-chloro-3-indonyl-S-galactoside) w/v in N,N, dimethyl-formamide
4 µl of 200 mg/ml IPTG (isopropyl ß-D-thiogalactopyranoside) w/v in sterile water
SOC to 100 µl

Ampicillin (amp)
Stock solution of 100 mg/ml in sterile water, kept in 500 µl aliquots at -20°C.

Electroporation of plasmids into E. coli was carried out using a Gene Pulser electroporator (BioRad) and 0.2 cm Gene Pulser electroporation cuvettes according to the following protocol:

1. The electroporation cuvettes and the sliding cuvette holder were chilled on ice.
2. The gene pulser apparatus was set to 25 mF capacitance, 1.8 kV voltage and 200 Ω resistance.
3. The electrocompetent cells were thawed on ice. To a microfuge tube containing 50 µl of competent cell suspension the appropriate amount of DNA for electroporation was added. Usually 10 µl of a ligation (approximately 50 ng), or approximately 10 ng of a stock plasmid were used.
4. The mixture of cells and DNA was transferred to a cold 0.2 cm electroporation cuvette, placed on the sliding holder and one pulse was applied at the above settings. This should result in a pulse of 12.5 kV/cm with a time constant of 4.5 - 5.0 msec.
5. 1 ml of ice cold SOC medium was immediately added to the cuvette and the cells were gently resuspended.

6. The cells were transferred to a 15 ml polypropylene pop-top tube (Falcon) and placed in an incubator shaker at 37°C for one hour to recover.

7. 100 ml of the cell suspension were plated on a 9 cm diameter LB agar (1.5% w/v) plate, containing appropriate selection agent (usually 100 µg/ml of amp). Grow the plate overnight at 37°C. The remainder of the cells was kept at 4°C.

7.4 Preparation of plasmid DNA

7.4.1 Preparation of large amounts of plasmid DNA

This method is based on that described by Birnboim and Doly, 1979.

Media, solutions, antibiotics

L-Broth medium (LBM)
per litre
10 g Bacto-tryptone
5 g Bacto-yeast extract
5 g NaCl
2 g $MgCl_2$

GTE solution
50 mM glucose
25 mM Tris.HCl pH 8.0
10 mM EDTA
autoclave, store at -4°C

High salt solution (pH 4.8)
60 ml 5 M potassium acetate
11.5 ml glacial acetic acid
28.5 ml water

1. A single colony was picked in a 1 litre flask containing 500 ml LBM with 100 µg/ml amp and incubated for approximately 16 h

(overnight) at 37°C shaking at 300 rpm (New Brunswick Scientific series 25 incubator shaker).

2. The bacterial cells were harvested by centrifugation at 4,000 rpm for 5 min at 4°C (Sorvall RC5C Du Pont centrifuge) and the supernatant was discarded.

3. The pellet was resuspended in 4 ml of ice cold GTE solution, transferred to a 50 ml polyallomer tube (Nalgene oak ridge centrifuge tube 31390050) and left for 5 min at room temperature.

4. 8 ml of freshly made 200 mM NaOH, 1% (w/v) sodium dodecyl sulphate (SDS) were added, shaken gently by inverting the tube several times and left on ice for 5 min.

5. To this 6 ml of ice cold high salt solution were added, mixed vigorously and left on ice for 5 min.

6. The mixture was centrifuged at 15,000 rpm for 15 min at 4°C (Sorvall RC5C centrifuge) and the plasmid containing supernatant was passed through a 0.45 micron syringe filter (Corning cellulose acetate membrane sterile acrylic filter).

After this step one of the 2 following procedures was followed:

7.4.1a Purification of closed circular DNA by centrifugation to equilibrium in cesium chloride-EtBr gradients.

1. For every ml of the supernatant 1.22 g/ml of CsCl and 200 µl of 750 µg/ml EtBr (ethidium bromide) were added.

2. The solution was mixed thoroughly and transferred to a Quick Seal polyallomer centrifuge tube (Beckman 342413). The reminder of the tube was filled with water containing 1.22 g/ml CsCl and 200 µl of 750 µg/ml EtBr per ml.

3. The plasmid preparation was centrifuged overnight at 55,000 rpm in a Beckman 27 Ultracentrifuge in a NVT65 rotor at 15°C.

4. Two bands of DNA were visualized by ordinary light; the lower one consisting of closed circular plasmid DNA was extracted by using a syringe with a 19 gorge needle.

5. The EtBr was extracted from the sample by using isopropanol saturated with CsCl and water.

6. The volume of the sample was measured, transferred to a 30 ml Corex tube and 2 volumes of water were added. The DNA was ethanol precipitated at room temperature for 1 h.

7. The sample was centrifuged for 15 min at 15°C in a Sorvall RC5C centrifuge and the DNA pellet was washed with 2 ml of 70% (v/v) ethanol.

8. The DNA was resuspended in 200 µl of TE and kept at -20°C.

7.4.1b Quick isolation of plasmid DNA

1. The supernatant was transferred to a Corex tube and the DNA was precipitated by addition of 0.8 volumes of isopropanol and standing for 1 h at room temperature.

2. The sample was centrifuged and the pellet was resuspended in 340 µl 0.5% (w/v) SDS in TE. This was transferred to an eppendorf tube and heated for 15 min at 90°C.

3. 113 µl of 10 M ammonium acetate were added and the mixture was left for 15 min, or overnight at -20°C before centrifugation for 5 min in a microfuge at 4°C.

4. The supernatant was transferred to another eppendorf tube and the pellet was discarded.

5. The plasmid DNA was ethanol precipitated from the supernatant (15 min at room temperature), washed with 70% (v/v) ethanol and the pellet was resuspended in 200 µl TE containing 10 mg/ml RNase A (DNase free).

6. After incubation at 37°C for 15 min the DNA sample was extracted twice with phenol : chloroform : isoamylalcohol 25 : 24 : 1, once with chloroform : isoamylalcohol 24: 1, then ethanol precipitated (see section 7.6.4) and resuspended in 200 µl of TE.

These methods gave up to 500-1 mg of plasmid DNA.

The DNA was quantitated by absorbance at 260 nm wavelength, assuming that 1 OD Unit = 50 µg/ml and by agarose gel electrophoresis (see section 7.5) against known standards.

7.4.2 Preparation of Plasmid DNA (medium scale)

Medium scale purification of plasmid DNA was prepared from a 25 ml bacterial culture grown in LBM overnight. A Qiagen Plasmid Purification Kit (Qiagen, tip-100 12143) was used to purify the plasmid DNA. This method is based on the optimised alkaline lysis method (Birnboim and Doly, 1979). Purification of the plasmid DNA by the Qiagen resin allows preparation of ultrapure plasmid DNA without the use of phenol, chloroform or EtBr. Approximately 75-100 µg of plasmid DNA were recovered.

7.4.3 Preparation of plasmid DNA (small scale)

1. A colony was picked into a 3 ml overnight culture (LBM medium, 10 µg/µl amp) and the bacteria were harvested in 1.5 ml eppendorf tube by centrifugation at 13,000rpm for 3 min.
2. The supernatant was discarded and the cells resuspended in 100 µl ice cold GTE.
3. 200 µl of freshly prepared 0.2 M NaOH, 1% (w/v) SDS were added, mixed by gentle inversion and the mixture was heated at 80-90°C for 10 min.
4. The tube was transferred on ice, 150 ml of high salt solution were added and mixed gently.
5. The mixture was centrifuged in a microfuge at 13,000 rpm for 10 min, and the supernatant recovered into a fresh eppendorf tube.
6. The DNA was ethanol precipitated, resuspended in 50 µl sterile water and left on dry ice for 5 min to precipitate residual SDS.
7. After centrifugation at 13,000 rpm, the supernatant was transferred to a fresh eppendorf tube.

This method gave approximately 20 µg of plasmid DNA.

7.5 Agarose gel electrophoresis

agarose gels

agarose (FMC BioProducts, Sea Kem Le Agarose, 50004)

usually 1% (w/v), or appropriate concentration, disolved by heating in

1 X TBE
5 µl of 1 µg/µl EtBr solution were added to 50 ml agarose gel solution

loading sample buffer (for DNA or RNA)
30% (v/v) glycerol in water
6 X TBE
50 µl/ml of bromophenol blue saturated solution

DNA size markers
1 kb ladder GIBCO-BRL, 15615-016
diluted in loading sample buffer to give final concentration 50 ng/µl

The gel tanks used for horizontal agarose gel electrophoresis were GIBCO BRL Horizon 58 1060BD (small size) or GIBCO BRL Horizon 11.14 1068BD (medium size).

7.6 General Cloning Techniques

7.6.1 Restriction digestion of DNA

Restriction enzymes and buffers were supplied by Boehringer Mannheim and Biolabs. The conditions recommended for DNA digestion by the Boehringer and Biolabs catalogue were generally followed. Usually 2 units of enzyme were used for 1 µg of plasmid DNA.

Digestions of genomic DNA were carried out as following:

10 µg genomic DNA
4 µl 0.1 M spermidine.HCl
10 µl 10 X restriction buffer
2 µl of enzyme (10 units/µl)
sterile water to 100 µl

The reaction was incubated for 2 h before another 40 units of enzyme were added and the incubation was continued overnight.

The products of DNA restriction digests were analysed by electrophoresis using agarose gels at appropriate concentrations in TBE buffer.

7.6.2 Elution of DNA fragments from agarose gels

The DNA fragment of interest was excised after electrophoresis from an agarose gel and the DNA was eluted by one of the following methods:

The Gelase kit (Cambio) was used for fragments over 1 kb according to the manufacturer's instructions. For smaller fragments the Geneclean II kit (Stratech Scientific 3106) was used according to the manufacturer's instructions, or the DNA was directly spun through siliconised glass wool (as described in *Trends in Genetics* 1992, 8:81).

7.6.3 Purification of DNA

For purification of DNA from enzymes, small DNA fragments (<175 nucleotides), mononucleotides and salts the "Wizard" Clean-up system (Promega A 7280) was used according to the manufacturer's instructions.

7.6.4 Ethanol precipitation of nucleic acids

DNA or RNA was precipitated by the addition of a 0.1 volume of 3 M sodium acetate (pH 5.2), followed by 2.5 volumes of -20°C ethanol. The mixture was held at -20°C for 1 h and the nucleic acids were pelleted by centrifugation, at 13,000rpm, 4°C. Small amounts of DNA (<1 µg) were precipitated as above, with the addition of 1 µl of glycogen (Boehringer 901393). To remove trace amounts of salt remaining from the precipitation, the nucleic acid pellet was washed in 1 ml of 70% (v/v) ethanol, recovered by centrifugation, dried in a vacum centrifuge

(Stratech Savant) and dissolved in an appropriate volume of sterile water or TE buffer.

7.6.5 Phenol-chloroform-isoamylalcohol extraction of DNA or RNA solutions

DNA or RNA solutions were phenol-chloroform-isoamylalcohol extracted to remove any contaminating proteins. The extraction solution (PCA) was phenol (Fisons T/p633/05) : chloroform : isoamylalcohol (25:24:1). Generally the DNA or RNA solutions were made up to at least 100 µl with sterile water or TE, an equal volume of PCA was added, the mixture was vortexed for 1 min and centrifuged for 5 min. The aqueous phase (upper) was transferred to a fresh eppendorf and re-extracted with PCA. Then an equal volume of chloroform:isoamylalcohol 24:1 was added, vortexed, microfuged and the aqueous phase was transferred to a new eppendorf and ethanol precipitated.

7.6.6 Dephosphorylation of linearised plasmid DNA

solutions

10 X STE
100 mM Tris.HCl pH 7.4
1 M NaCl
10 mM EDTA

Removal of the 5' terminal phosphate groups of linearised plasmid DNA with identical termini was required to prevent recircularisation of the plasmid DNA in subsequent ligation steps.

1. After restriction digestion of DNA, the volume was made up to 50 µl with TE and 5 units of alkaline phosphatase (Boehringer, 713023) were added directly, and incubated at 37°C for 45 min.

2 The reaction was stopped by the addition of 2.5 µl 10% (w/v) SDS and 5µl 10 X STE and the sample was incubated for 15 min at 70°C.

3. An equal volume of phenol:chloroform 1:1 was added, vortexed briefly, and centrifuged at 13,000 rpm before recovering the aqueous phase.

4 The DNA was ethanol precipitated and resuspended in TE .

7.6.7 Ligation of DNA fragments

solutions

10 X ligation-kinase buffer
660 mM Tris.HCl pH 7.4
10 mM spermidine.HCl
100 mM $MgCl_2$
150 mM dithiothreitol (DTT)
2 µg/µl nuclease-free bovine serum albumin (BSA)

store at -20°C in 200 µl aliquots

Generally 1:3 molar ratio vector DNA: insert DNA was used for the ligations. A control ligation containing no insert DNA was always included to assess the extent of self-ligation. 100-200 ng of vector was the optimal concentration for a 10 µl reaction, assembled as following:

1 µl 10X ligation-kinase buffer
1 µl 10 mM ATP
1 µl (1u/µl) T4 DNA ligase (Boehringer, 716 359)
vector DNA (usually 100-200 ng)
insert DNA (3 X molar excess)
sterile water to 10 µl

The reaction components were assembled fresh each time. The reaction was left to proceed overnight at 15°C, or for 2 h at room temperature (approximately 25°C).

The reaction was ethanol precipitated and the DNA was resuspended in 20 µl of sterile water. Half of that was electroporated in competent cells.

7.6.8 Blunt-ending DNA fragments

solutions

10 X T4 DNA polymerase buffer
50 mM Tris.HCl pH 8.5
15 mM ammonium sulfate
7 mM $MgCl_2$
0.1 mM EDTA
10 mM ß-mercaptoethanol
200 µg/ml BSA
store in 200 µl aliquots at -20°C

With this reaction protruding 5' ends of double stranded DNA that resulted after restriction digestion, were filled in by the T4 DNA polymerase (5' to 3' polymerase activity) and protruding 3' ends were recessed (3' to 5' exonuclease activity). The reaction was assembled as following:

2 µl dNTPs 2.5 mM each
5 µl T4 DNA polymerase buffer
42 µl of DNA fragment (usually up to 5 µg)
1 µl (1u/µl) T4 DNA polymerase (Boehringer 1004 786)

The reaction was incubated at 37°C for 1 h before it was ethanol precipitated. The DNA was resuspended so as to give 400 ng/µl.

7.6.9 Phosphorylation of nonphosphorylated linkers and ligation to DNA fragment

Before adding linkers to a DNA fragment it was first necessary to phosphorylate their 5' ends. The reaction was assembled as following:

1 µl 10 X ligation-kinase buffer
1 µl 10 mM ATP
2 µg nonphosphorylated linkers (approximately 700 pmol)

0.2 µl (10 u/µl) T4 DNA polynucleotide kinase (Boehringer 174 645)
sterile water to a final volume of 10 µl

The reaction was incubated at 37°C for 1 h and was used directly for ligation to the DNA fragment. The following components were added to the 10 µl reaction:

1 µl ligation-kinase buffer
1 µl 10 mM ATP
1 µl blunt-ended DNA fagment (400 ng)
2 µl (1u/µl) DNA T4 DNA ligase
sterile water to 20 µl

The reaction was incubated overnight at 15°C, or at room temperature for 2 h. Subsequently it was ethanol precipitated and resuspened in 5 µl sterile water.

7.6.10 Phosphorylation of adaptors and ligation to DNA fragment

To 10 pmol ends of DNA fragment a 20 X molar excess of oligonucleotide adaptors was added (100 pmol), as following

200 pmol adaptor(1)
1 µl 10 X kinase-ligase buffer
1 µl 10 mM ATP
0.1µl T4 polynucleotide kinase
sterile water to 10 µl

The reaction was incubated at 37°C for 1 h, then for 5 min at 70°C and chilled on ice. To that 200 pmol of adaptor(2) and sterile water to 20 µl were added. The mixture was incubated for 10 min at 70°C and left to cool at room temperature.

The phosphorylated adaptors (final concentration 10 pmol/µl) were used for the ligation to the DNA fragment as following:

10 pmol. of DNA ends

10 µl (100 pmol) adaptors
10 µl 10 X ligation - kinase buffer
10 µl BSA (1 mg/ml)
10 µl (1u/µl) T4 DNA ligase
sterile water to 100 µl

The ligation was incubated for 2 h at room temperature or at 15°C overnight. The ligase was inactivated by heating at 70°C for 5 min. Subsequently the cloned adaptors were phosphorylated by adding the following components to the 100 µl reaction:

8 µl 10 mM ATP
1 µl 10 X ligation-kinase buffer
1 µl (10 u/µl) T4 DNA polynucleotide kinase

The reaction was incubated at 37°C for 1 h and then at 70°C for 5 min. The sample was loaded on a 2% (w/v, agarose in TBE) agarose gel and the band was eluted in a volume that would give 50 ng/µl.

7.6.11 Polymerase Chain Reaction (PCR)

When the template for the amplification of the fragment of interest was plasmid DNA, 20 ng of DNA were used and when the template was genomic DNA, 500 ng of DNA were used.

The reaction was assembled as following:

0.5 µl (5 u/µl) Taq DNA polymerase (Promega M1862)
5 µl 25 mM $MgCl_2$ (provided by Promega)
5 µl 10 X PCR buffer (Provided by Promega)
2 µl dNTP mix 2.5 mM each
50 µM primer(1)
50 µM primer(2)
sterile water to 50 µl

The reaction was placed in the PCR machine which was set at the following temperatures (unless otherwise stated):

95°C for 2 min (melting temperature)
55°C for 1 min 30 sec (annealing temperature)
72°C for 2 min (extension temperature)

This was repeated for 30 cycles and followed by 1 cycle of:

95°C for 2 min
55°C for 1 min 30 sec
72°C for 10 min
before placed on ice.

A 5 µl aliquot of the reaction was run on a 1% agarose gel to visualise the products of the reacion.

The annealing temperature could vary (50°C-60°C) depending on the stringency required.

7.6.12 Reverse transcription PCR (RT-PCR)

Approximately 500 ng to 1 µg of total RNA were used for the reverse transcription reaction. The components were assembled as following:

2 µl 25 mM $MgCl_2$
2 µl 10 X PCR buffer
2 µl dNTP mix 10 mM each
0.5 µl (20U) RNA guard (Pharmacia 27 0815 01)
1 µl (100 pmol, 50u/µl) random hexamer primers (Pharmacia, 27 2166 01)
1 µl (12u/µl) Moloney Murine Leukemia Virus Reverse Transcriptase (Pharmacia, 27 0925 02)
sterile water to 20 µl

The reaction was placed in the PCR machine and incubated at the following temperatures for 1 cycle:

10 min at 23°C
45 min at 42°C
5 min at 95°C

After placing on ice the following components were added for the PCR step:

8 µl 25 mM $MgCl_2$
8 µl 10 X PCR buffer
1 µl 50 µM primer (1)
1 µl 50 µM primer (2)
0.5 µl (5u/µl) Taq DNA polymerase
water to 100 µl

At this point the temperatures described before for the PCR amplification were used.

7.6.13 Cloning of PCR fragments

When primers including specific restriction sites for cloning were used for the PCR amplification, the PCR product was digested with the equivalent restriction enzymes to generate the ends. The restriction sites were never placed at the end of the primers because most restriction enzymes do not cut at the end of a fragment. At least 3 extra nucleotides were included in the primers beyond the restriction sites.

Taq DNA polymerase has a terminal transferase activity adding a 3' A-overhang to each end of the PCR products. A vector with a T overhang was therefore used for direct cloning of PCR fragments (TA cloning kit, Invitrogen, K2030-01).

7.7 Genomic DNA extraction

solutions

Proteinase K (PK) buffer:
50 mM Tris.HCl pH 7.5
100 mM EDTA
50 mM NaCl (optional)
0.5% SDS

Proteinase K (PK)
10 mg/ml in sterile water

7.7.1 from tissues

1. 100 µg of tissue were digested at 55°C overnight in 1 ml (10 volumes) of PK buffer containing 0.5 mg/ml PK.

2. The sample was extracted once with phenol, the aqueous phase was transferred to a fresh eppendorf and then PCA extracted.

3. The aqueous phase was transferred to a new eppendorf and the DNA became visible as white strings after the addition of 2.5 volumes of ethanol. At that stage it was picked with the tip of a Pasteur pipette, which was made into a hook.

4. The DNA which adhered to the pipette was transferred to an eppendorf containing 70% ethanol and the pipette was shaken until the DNA fall in the solution.

5. After a spin at 13,000rpm for 3 min the pellet was dried in a vacuum microfuge, 400 µl of TE buffer were added and it was left to resuspend overnight at 4°C.

6. 100 µg/ml of RNase (DNase free) were added and the sample was incubated at 37°C for 1 h.

7. The sample was PCA extracted, ethanol precipitated and resuspended in 200 µl TE at 4°C overnight.

When the tissue for the DNA extraction was mouse tail, 1 cm approximately of mouse tail was cut off in 500 µl PK buffer containing

0.5 mg/ml PK and left at 55°C overnight. The same procedure was applied to mouse ear-punches, but the volume of PK buffer added was 100 µl. At this point either of the following two procedures were followed:

7.7.1a Preparation of DNA for Southern blot analysis

Procedures 2-7 were followed as described above (section 7.7.1).

7.7.1b Preparation of DNA for dot/slot blot analysis

150 µl, or 30 µl of 5 M NaCl were added to 500 µl PK-treated mouse tails, or ear-punches respectively, vortexed for 5 min, then equal volume of chloroform was added and vortexed. Most of the supernatant would be stored at -4°C and 50 µl would be further incubated with 150 µl 0.53 M NaOH (0.4 M final concentration) for 30 min at 37°C.

7.7.2 from cells grown in tissue culture

1. Cells from a 9 cm culture dish (10^6-10^7 cells), were washed twice with ice cold PBS and scraped with a rubber policeman in 1 ml of PBS.
2. After being transferred to an eppendorf tube, they were centrifuged for 3 min at 13,000rpm and resuspended in 500 µl PK buffer containing 0.5 mg/ml PK.
3. The sample was incubated for 3 h at 55°C followed by procedures 2-7 described for the extraction of genomic DNA from tissues.

7.8 Total RNA extraction

DEPC (diethyl pyrocarbonate) treatment of solutions (any solution that does not contain Tris base): add DEPC 1:1000 (v/v) and autoclave.

For the extraction of total RNA from tissue or cells grown in cell culture the RNAzol B method (8050-4014, Biogenesis) was used.

1. Approximately 50 mg of tissue was homogenised in a glass Dounce homogenizer with 1 ml RNAzol B, before being transferred to an eppendorf tube. Cells grown in monolayer were lysed directly on the culture dish by the addition of RNAzol B (1 ml for 10^6 cells) and then transferred to an eppendorf tube.

2. 100 µl (1:10 v/v) of chloroform were added and vortexed for 15 sec before standing on ice for 5 min.

3. The samples were centrifuged for 15 min at 13,000 rpm.

4. The aqueous phase was transferred to another eppendorf and an equal volume of isopropanol was added. The sample was left on ice for 30 min or kept at -20°C overnight.

5. The sample was microfuged at 13,000 rpm for 15 min, the RNA pellet was washed with 70% (v/v) ethanol, dried and resuspended in 30-50 µl DEPC-treated water.

6 Quantitation was done by absorbance at 260 nm, assuming that 1 OD=40 µg/ml and by agarose gel electrophoresis against known standards.

7.9 mRNA extraction

mRNA was purified by the mRNA purification kit (Pharmacia 27-9258-01), from total RNA prepared previously from tissue or cells by the RNAzol method, following the instructions of the manufacturers.

7.10 *In vitro* transcription

1. CsCl purified DNA was used to produce linear DNA template by restriction digest with enzyme that cuts at the 3' end of the DNA fragment to be transcribed. An aliquot of the reaction was run on an agarose gel to ensure that restriction had gone to completion. The remainder of the reaction was PCA extracted and ethanol precipitated.

The DNA was resuspended in DEPC treated water at a concentration of 1 µg/µl.

2. *In vitro* transcription was carried out using T3 RNA polymerase (Promega P2083) The reaction was assembled as following:

2 µl 5 X Transcription buffer (Promega)
1 µl RNA guard
0.5 µl 100 mM DTT
2 µl dNTP mix 2.5 mM each
1 µl DNA template (1 µg)
3 µl T3 RNA polymerase 20 u/µl

The reaction was incubated at 37°C for 1 h and then DNase I was added (10u) and the reaction was incubated for another 30 min. The volume was made up to 50 µl with sterile DEPC-treated water and the sample was purified through a sterile DEPC-treated G-50 Sephadex column. 1 µl of the purified sample was run on an agarose gel and the rest was precipitated by the addition of 1 volume isopropanol and 1/10 volume of 3 M amonium acetate. The sample was left for 1 h on ice then centrifuged at 13,000rpm, washed with 70% (v/v) ethanol and resuspended in 5 µl sterile DEPC-treated water.

7.11 Preparation of G-50 Sephadex columns for purification of *in vitro* prepared RNA

1. DEPC-treated 10 X STE (100 mM Tris.HCl pH 7.5, 10mM EDTA, 2 M NaCl) was made as following:

DEPC treat 10 mM EDTA, 1 M NaCl in water (leave overnight before autoclaving), then add Tris base (to 100 mM), adjust the pH to 7.0 with HCl, autoclave again.

2. 2 mg of G-50 sephadex was added to 35 ml DEPC-treated water and autoclaved. To that 4 ml 10 X STE (final concentration 1 X) was added.

3. The columns were made in 1 ml syringe barrels by using sterile Whatman filters and G-50 Sephadex in 1 X STE. The column was centrifuged at 2,000 rpm for 5 min.

7.12 Dideoxy-termination sequencing of double-stranded DNA templates and cycle sequencing (Sanger *et al.*, 1977).

1. For standard sequencing approximately 2 µg of uncut plasmid DNA was denatured by incubating in 200 mM NaOH for 5 min at room temperature. The DNA was precipitated with 1/5 volume of 10 M ammonium acetate and 4 volumes of absolute ethanol at -20°C for 15 min.

2. The DNA precipitate was washed with 1 ml of 70% (v/v) ethanol, dried and resuspended in 7 ml of water.

3. The sequencing reactions were prepared using the Sequenase Version 2.0 kit (USB, 70770) according to the manufacturers instructions.

Direct cycle sequencing of DNA fragments was done with Vent polymerase (New England Biolabs, 2575) according to the manufacturer's instructions.

7.13 Sequencing gel

Sequencing products were electrophoresed through a 0.2 mm 6% (v/v) denaturing (i.e. 8 M urea) acrylamide gel in TBE.

1. The gel was made using solutions obtained from the 'Sequagel' kit of National Diagnostics. For a 50 ml gel the following quantities were used:

14.4 ml concentrate
39.6 ml diluent
6 ml buffer
0.48 ml 10% (v/v) ammonium persulphate (APS)
50 µl N,N,N',N'-tetramethyl-ethylenediamine (TEMED)

2. The gel was poured between glass plates, one of which had been siliconised and an appropriate comb was used to create the wells *in situ*.

3. Samples were denatured at 90°C for 10 min and then 2 µl of each was loaded into the wells in a defined order (A-C-G-T was used here).

The gel was subjected to electrophoresis (30-40 W, 2000 V for 3 h or more.

4. After electrophoresis, the gel was soaked in 10% (v/v) methanol, 10% (v/v) acetic acid for 15 min to leach out the urea and to fix the DNA. The gel was transferred to 3MM Whatmann paper and dried at 80°C under vacuum using an ice-trap to condense the evaporating fix solution.

5. Sequencing products were visualised by autoradiography using Kodak BioMax film.

7.14 Southern Blot

This method was based on that described by Southern, 1975.

solutions

acid depurination solution
0.25 M HCl

denaturing solution
0.5 M NaOH
1.5 M NaCl

neutralising solution
1 M Tris.HCl pH 7.4
1.5 M NaCl

1. The samples were electrophoresed on a 1% agarose gel containing 0.5 mg/ml EtBr.

2. The gel was photographed and then acid depurinated for 20 min.

3. The gel was rinsed with distiled water and then denatured for 30 min.

4. Following denaturation the gel was neutralised 2 X 5 min.

5. The gel was rinsed briefly in 20 X SSC and then transferred to a vacuum blotting device (Pharmacia LKB VacuGene XL). The membrane

used in all transfers was Hybond-N (Amersham RPN.303N) and it was prewet in distiled water and 20 X SSC. The transfer buffer was 20 X SSC.

6. After the transfer, the filter was rinsed briefly in 2 X SSC and then UV crosslinked on a UV Crosslinker (Stratagene Stratalinker 1800) at 1,200 μJoules.

7.15 Dot/slot blot

A Hybond-N filter was cut at the size of the dot blot apparatus and prewet in 1 X TBE and then in 0.4 M NaOH for 5 min. 3MM Whatman paper was soaked in 0.4 M NaOH and placed under the Hybond-N filter. The apparatus (BioRad, Bio-Dot apparatus) was assembled following the manufacturer's instructions.

After preparation of DNA samples (200μl) from mouse tails, or ear-punches for dot blot analysis (section 7.7.1b), they were loaded into the slots of the assembled apparatus and left for 30 min before vacuum was applied.

7.16 Northern Blot

solutions

20 X FRB
0.4 M MOPS (3-[N-morpholino]propanesulphonic acid).NaOH pH 7.0
100 mM sodium acetate
10 mM EDTA (pH 8.0)

denaturing solution
50 mM NaOH
10 mM NaCl

neutralising solution
100 mM Tris.HCl pH 7.5

sample buffer
1 X MOPS
50% (v/v) formamide
7% (v/v) formaldehyde
0.5 mg/ml EtBr
bromophenol blue

1. RNA samples were ethanol precipitated.

2. The RNA samples were resuspended in sample buffer and heated at 65°C for 15 min before being loaded onto a1% agarose gel containing 1 X FRB and 7% (v/v) formaldehyde.

3. The gel was electrophoresed for 2 h at 100 V/cm, until the bromophenol blue was near the end of the gel.

4. After electrophoresis and photography, the gel was denatured for 20 min, neutralised for 10 min, rinsed 3 X sterile water and blotted. The vacum transfer of RNA to a Hybond-N (Amersham RPN.303N) membrane was exactly the same as for DNA.

5. The size markers used were obtained from GIBCO-BRL (15620-016). When total RNA was used, the 28S and 18S ribosomal RNA would also serve as a size indicator (4.7 kb and 1.9 kb respectively).

7.17 Radiolabelling of DNA

7.17.1 Oligonucleotide-labeling of DNA fragments

This method is based on that described by Fienberg and Vogelstein, 1984.

solutions

5X labeling mix
250 mM Tris.HCl pH 7.5
25 mM $MgCl_2$
10 mM DTT
1 mM Hepes.HCl pH 6.6
26 u/ml random hexamer primers (Pharmacia 27-2166-01)

dNTP mix
500 µM dATP
500 µM dGTP
500 µM dTTP

DNA fragments for radiolabeling were firstly excised and eluted from agarose gels.

1. 10-25 ng of DNA in a final volume of 28.5 µl of water was denatured by boiling for 5 min.

2. Tubes were placed on ice, then centrifuged briefly in a microfuge to collect the contents to the bottom.

3. The following 50 µl reaction was assembled:

28.5 µl DNA
10 µl labeling mix
2 µl 10 mg/ml BSA
2 µl dNTP mix
5 µl [α-^{32}P] dCTP 50 mCi (3,000 Ci/mmol) (Amersham AA0005)
2 µl (2 u/µl) Klenow fragment of E. coli DNA polymerase I (Boehringer
1008 412)

4. The reaction was incubated at room temperature for 2-3 h, or for 1 h at 37°C. .

5. Labeled DNA was purified from unincorporated [α-^{32}P]-dCTP using the a G-50 sephadex spin column.

Lately the 'High Prime' kit (Boehringer 1585 592) was used according to the manufacturer's instructions.

7.17.2 T4 polynucleotide kinase-labeling of 5' termini.

Oligonucleotides may be labeled directly using this method, however DNA restriction fragments require prior dephosphorylation.

The procedure described for phosphorylating linkers (section 7.6.9) was followed in both occasions but instead of ATP, 50 mCi of [γ-^{33}P]ATP (NEN NEG-302H) were added. The kinase was subsequently inactivated by heating at 68°C for 10 min. The labeled oligonucleotide was purified by ethanol precipitation in the presence of glycogen.

7.17.3 Terminal Transferase tailing of 3' termini

This method was used to label oligonucleotides with a tail of ^{35}S for *in situ* hybridisation.

1. 10 ng of oligonucleotide was tailed in a 20 µl reaction. The following were added in order:

3 µl sterile water
2 µl oligonucleotide (0.5 - 1.0 nmol)
4 µl 5 X terminal transferase buffer (Boehringer)
6 µl 5 mM $CoCl_2$
25 units terminal transferase (Boehringer 220 582)
40 µCi [α-^{35}S] dATP (1,000 Ci/mmol) (Amersham SJ1304)

2. The reaction was incubated at 37°C for 1 h.
3. 80 µl of TE and 20 mg yeast tRNA (Boehringer 109495) were added to quench the reaction.
4. The sample was PCA precipitated, the pellet was dried and resuspended in 50 ml TE. If the labeled oligonucleotide was to be used for *in situ* hybridisation, the TE for resuspension contained 10 mM DTT.

2 µl would be mixed in a scintillation vial with 3 ml scintillation fluid (Ultima gold, Packard 6013329) and in order to determine the incorporation of radioactivity.

7.17.4 Synthesis of riboprobes

Riboprobes were prepared from the 250 bp SV40 sequence cloned in modified pBluescriptII (ΔPS.N.A construct, appendix 2). Antisense SV40

probe was synthesised with T3 RNA polymerase after XhoI digestion of the plasmid and sense SV40 probe was synthesised with T7 RNA polymerase after KpnI digestion of the plasmid.

6 µl 5 X Transcription buffer (Promega)
2 µl RNA guard
1 µl 1 M DTT
1 µl 10 mM CTP
1 µl 10 mM ATP
1 µl 10 mM GTP
10 µl (~125 µCi) [α-^{35}S] UTP (Amersham SJ1381)
5 µl linear DNA template (~5 µg)
3 µl T3 RNA polymerase 20 u/µl (Promega P2083)
(or, 3 µl T7 RNA polymerase 20 u/µl (Promega P2075))
sterile DEPC-treated water to 30 µl

The reaction was incubated for 30 min at 37°C before 2 µl tRNA (10 mg/ml) and 1 µl DNase were added.

After 10 min incubation at 37°C, 1 µl of 200 mM EDTA and TE (with 50 mM DTT) to a final volume of 100 µl were added.

The reaction was extracted once with phenol then twice PCA extracted and 10 µl of 3 M sodium acetate were added followed by ethanol precipitation.

The pellet was washed 1 X 80%(v/v) ethanol/50 mM DTT and 1 X 100% ethanol. After drying it was resuspended in appropriate volume of TE/50 mM DTT to give approximately 1 X 10^6 cpm/ml.

7.18. Hybridisation Conditions (Southern and Northern analysis)

Hybridisation conditions used were based on those described by Church and Gilbert, 1984.

1M $NaHPO_4$ pH7.0
72 g anhydrous Na_2HPO_4
4 ml H_3PO_4 (85% v/v)
up to 1 litre with sterile water

Hybridisation Buffer:
0.25 M $NaHPO_4$ pH 7.2
1 mM EDTA
7% (w/v) SDS
1% (w/v) BSA

Wash Buffer I:
20 mM $NaHPO_4$ pH 7.2
2.5% (w/v) SDS
0.25% (w/v) BSA
1 mM EDTA

Wash Buffer II:
20 mM sodium phosphate pH 7.2
1 mM EDTA
1% (w/v) SDS

1. Filters were pre-wet in distilled water and then in 2 X SSC (section 7.1).

2. The hybridisation was performed in a rotating glass cylinder (Techne Hybridiser ovens). 10 ml of Hybridisation Buffer were added to the cylinder with the filter.

3. Prehybridisation and hybridisation were carried out at 65°C unless otherwise specified.

4. The filters were prehybridised for 1 h, after which the probe was added directly and hybridisation proceeded overnight. (Double-stranded probes were denatured by boiling for 5 min and then placed on ice).

5. Washes were performed at 65°C (unless otherwise stated) with 2 changes of Wash Buffer I for 10 min each, followed by three changes of Wash Buffer II each for 20 min.

6. The filters were blotted dry, but not allowed to dry out, then placed between Saran wrap, and against X-ray film for autoradiography.

7.19 Colony screening - identification of recombinants

Some plasmids used for cloning (i.e. pBluescriptIIKS-, Stratagene) contain a segment of E. coli DNA encoding for ß-galactosidase (lacZ gene). Such plasmids form dark blue colonies when plated in the presence of the chromogenic substrate, X-gal and inducer, IPTG. The multiple cloning site (MCS) of these plasmids is upstream of the lacZ gene and therefore cloning of a foreign DNA fragment would result in a shift of the reading frame and loss of ß-galactosidase activity (formation of white colonies). After electroporation with such plasmids the cells woud be plated on LB agar/X-gal plates (containing 100 µg/ml amp) and incubated over night at 37°C. The white colonies would be picked and DNA would be prepared by the small scale plasmid preparation method (section 7.4.3).

Trying to identify positive recombinant colonies after cloning attempts by small scale plasmid preparation was not always possible, due to the number of colonies to be screened, or the absence of reporter gene in the plasmid used for cloning. In that case colony hybridisation was used as an alternative.

Colonies to be screened were replated on LB-amp plate using a circular paper grid and grown overnight at 37°C.

A circular nitrocellulose filter was laid on to the plate for 20 sec, then removed and laid on to a new LB-amp plate which was incubated at 37°C overnight so as to create a replica. Another nitrocellulose filter was laid on the original plate to create a duplicate. Both filters were treated as following:

3 min in 10% (w/v) SDS
5 min in 1.5 M NaCl, 0.5 M NaOH
5 min in 1.5 M NaCl, 0.5 M Tris.HCl pH 7.5

The filters were washed thoroughly in 2 X SSC and UV crosslinked before they were left to air dry. The hybridisation of the filters was the same as for Southerns and Northerns.

7.20 Stripping of filters

Filters that needed to be reprobed were stripped by placing them in boiling 0.1% (w/v) SDS and leaving the solution to cool to room temperature. This was repeated if necessary until no signal could be detected using the Geiger counter. Stripped filters were exposed to autoradiographic film at least overnight before reprobing, to ensure that all previous signals had been removed.

7.21. Protein manipulation

7.21.1 protein extraction

solutions

Lysis buffer I
100 mM NaCl
10 mM Tris.HCl pH 7.8
10 mM EDTA
0.5% (w/v) sodium deoxycholate
0.5% (v/v) Nonidet P-40

Lysis buffer II
0.5% (v/v) Nonidet P-40
0.5% (w/v) sodium deoxycholate
PBS

PMSF solution
phenylmethanesulfonyl fluoride (PMSF) 1% (v/v) in isopropanol

7.21.1a from tissues

Mouse tissues were kept on dry ice until they were homogenised in lysis buffer II.

1. Approximately 100 mg of tissue was weighed and placed in a Dounce homogenizes with 900 µl of lysis buffer II and 10 µl of PMSF solution.

2. After homogenization the sample was transferred to a 1.5 eppendorf tube on ice and sonicated 4 X 1 min at 24 microns with a Soniprep 150 MSE sonicator.

3. The sample was centrifuged at 3,000 rpm for 5 min and the supernatant was precipitated with 4 volumes of methanol for at least 1 h.

4. After 10 min centrifugation at full speed the pellet was resuspended in 200 µl lysis buffer.

5. The sample was kept at -20°C in 50 µl aliquots.

7.21.1b from cells

1. Tissue culture cells from a 9 cm culture dish (10^6-10^7 cells) were washed 3 times in ice cold PBS and then lysed with 1 ml of ice cold lysis buffer I containing 10 µl PMSF solution.

2. The cells were left at room temperature for 5 min with occasional rocking, scraped with a rubber policeman and transferred to an eppendorf tube.

3. The sample was passed several times through a 26 gauge needle and left for 10 min on ice with occasional vortexing.

4. Nuclei and insoluble debris were removed by 5 min centrifugation at 3,000 rpm.

5. The supernatant was transferred to a new eppendorf and precipitated with 4 volumes of methanol at -20°C for at least 1 h.

6. Samples were centrifuged at 13,000 rpm for 15 min at 4°C and the pellet was resuspended in a small volume of lysis buffer I.

7.21.2 Quantification of protein concentration by using the BioRad protein assay

1. 5 µl of the protein sample to be assayed were added to 800 µl of water in a 1 ml plastic UV cuvette. 200 µl of BioRad protein assay reagent (BioRad 500-0006) were added and mixed thoroughly.

2. The sample was incubated at room temperature for 10-15 min and the absorbance was read at 595 nm.

3. 4 reactions containing 5, 10, 15, and 20 µg of BSA were set al.,ong with the samples and used as a standard.

7.21.3 SDS - PAGE electrophoresis

solutions

The acrylamide used was either Protogel (National diagnostic EC-890) or prepared as following:

acrylamide stock 30% T 2.67%C
29.2 g acrylamide
0.8 g bisacrylamide (N,N'-methylenebisacrylamide)
make up to 100 ml with distilled water
wrap in foil and store in the fridge

sample buffer
4% (w/v) SDS
20% (v/v) glycerol
125 µM Tris.HCl pH 7.4
10% (v/v) ß-mercaptoethanol added just before use
0.001% (w/v) bromophenol blue

running buffer
1.5 M Tris.HCl pH 8.8

stacking buffer
0.5 M Tris.HCl pH 6.8

electrode buffer
25 mM Tris.HCl pH 6.3
192 mM glycine
0.1% (w/v) SDS

10% (w/v) ammonium persulphate (APS)
0.1 g in 1 ml sterile water

Proteins isolated from tissues, culture cells, or generated by in vitro translation were examined for size heterogeneity by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A BioRad 'mini Protean' or a BioRad 'Protean II Xi cell' gel systems were used.

10% or 15% slab gels, with 6% stacking gels were generally used and the ingredients are given below:

separating gel	10 X 7 cm 10%	10 X 7 cm 15%	16 X 16 cm 15%
H_2O	1.4 ml	2.4 ml	4.7 ml
running buffer	1.5 ml	1.5 ml	5 ml
acrylamide	2 ml	3 ml	10 ml
10% SDS	0.06 ml	0.06 ml	0.2 ml
10% APS	20 µl	20 µl	0.16 ml
TEMED	3 µl	3 µl	5 µl
total	6 ml	6 ml	20 ml

stacking gel	10 X 7 cm	16 X 16 cm
H_2O	1.53 ml	5.7 ml
stacking buffer	0.63 ml	2.5 ml
acrylamide	0.33 ml	2 ml
10% SDS	25 µl	0.1 ml
10% APS	12 µl	0.1 ml
TEMED	2 µl	5 µl
total	2.5 ml	10 ml

10-20 µl of protein sample was mixed with 1 volume sample buffer, boiled for 10 min and electrophorised at 20 mA through the stacking gel and 40 mA through the separating gel.

3 µl of prestained protein molecular weight standards (GIBCO-BRL 26041-020) were mixed with equal volume of sample buffer boiled for 10 min and loaded along with the samples.

An overnight electrophoresis on the big gel was carried out at 40 V through the stacking gel and at 70 V through the separating gel.

7.21.4 Gel staining

Coomassie Blue solution
100 ml methanol
20 ml acetic acid
80 ml distilled water
0.02 g Coomassie Blue

In order to visualize all proteins contained in the samples the gel was stained with Coomassie Blue solution and destained for several hours in a solution of 10% (v/v) methanol, 10% (v/v) acetic acid.

7.21.5 Gel fixing-amplifying-exposing

Gels bearing radiolabeled proteins were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 15 min and rinsed several times with distilled water. Then they were treated with fluorographic reagent Autofluor (National Diagnostics, LS-315) containing 0.5% glycerol for 1 h and dried on 3MM Whatman paper on a vacuum gel drier for 1 h and exposed on X-ray film.

7.21.6 Immunoblotting

The gel was transferred to a nitrocellulose filter and probed with anti-PrP primary antibodies and peroxidase conjugated secondary antibodies as described below.

7.21.6a Transfer

transfer buffer
5.8 g Tris base
2.9 g glycine
3.7 ml 10% (w/v) SDS
200 ml methanol
make up to 1 l with water

1. 6 pieces of 3MM Whatman paper were cut out exactly to the size of the gel and soaked in transfer buffer.
2. A piece of nitrocellulose (Millipore immobilon-p IPVH00010) cut out to the exact size of the gel was soaked in methanol for 2 min and then in distilled water for 5 min.
3. The gel was rinsed with distilled water.
4. These were loaded on the transfer apparatus in this order:

   3 Whatman 3MM papers
   gel
   nitrocellulose filter
   3 Whatman papers

5. The transfer was done at 10 V, 80-100 mA for 1 h.
6. The apparatus was unloaded and the filter allowed to dry slightly.
7. The filter was rewet in methanol and then PBS (section 7.1).

7.21.6b Western Blotting

block solution
3% (w/v) BSA in PBS

1. The filter was washed shaking in block solution for at least 3 h.
2. The block solution was poured of and the filter was washed twice with PBS.
3. The filter was placed in a plastic bag cut to the same size as the filter and 2 ml 3% (w/v) BSA in PBS containing the primary antibody at appropriate dilution were added. Usually the mouse anti-hamster PrP monoclonal antibody 3F4 (Kascsak *et al.*, 1987) was used at 1:500 dilution, or the rabbit anti-PrP polyclonal antibodies 1B3 and A8 (Farquhar *et al.*, 1989) were used at 1:1,000 dilution. The filter was incubated in this solution shaking overnight.
4. The filter was washed 4 X 5 min in PBS.
5. The filter was incubated with the secondary antibody: peroxidase conjugated rabbit IgG (SIGMA, A 9169), or mouse IgG (SIGMA, A 9044) diluted (usually 1:2,000) in 3% (w/v) BSA in PBS, for 1 h.
6. The filter was washed 4 X 5 min in PBS.
7. The filter was over-laid with the substrate solution (3,3' diaminobenzidine tetrahydrochloride, SIGMA FAST DAB tablets D4293) to allow the color (brown) to develop.
8. The filter was washed several times in PBS to stop the reaction.

7.22.1 Metabolic radiolabeling of cellular proteins.

1. 48 h after transfection of the cells, the medium was drawn off and they were washed twice with DMEM (see section 7.29.1) lacking methionine and cysteine (Gibco).
2. The cells were incubated with 3 ml DMEM minus methionine-cysteine for 1 h.
3. This medium was replaced with 1 ml DMEM minus methionine-cysteine supplemented with [^{35}S]-methionine at 200 µCi/ml (Tran ^{35}S label ICN 51006) and the cells were incubated for 2 h.

4. The cells were lysed in 1 ml lysis buffer I and the protein extraction procedure from cells (7.21.1b) was followed up to the methanol precipitation step.

7.22.2 Pulse-chase

1. The cells were incubated with 3 ml DMEM minus methionine-cysteine for 1 h.

2. This medium was replaced with 1 ml DMEM minus methionine-cysteine supplemented with [^{35}S]-methionine at 100 µCi/ml (Tran ^{35}S label ICN 51006) and the cells were incubated for 1 h.

3. The medium was replaced with DMEM and the cells incubated for different duration of time before they were lysed.

7.23 Immunoprecipitation (ip) of PrP

This method is based on that described by Borchelt *et al.*, 1993 and by Dr S.A. Priola (personal communication).

Buffers

DLPC

100 mg of L-a-lecithin (Phosphatidylcholine SIGMA P-3556)
1.3 ml Tris.HCl pH 7.5
21.3 ml sterile saline (NaCl 0.8% (w/v))
2.5 ml 20% (w/v) Sarcosyl

The buffer was sonicated for 5-10 min, until the lecithin went into solution. 250 µl of PMSF solution (see section 7.21.1) were added. The solution was stored at 4°C.

TN buffer + 1% (w/v) Sarkosyl

25 ml 1 M Tris.HCl (pH 7.0)
50 ml 5M NaCl
25ml 20% (w/v) Sarcosyl

The volume was made up to 500 ml with water and filtered.
Stored at 4°C.

Protein-A-sepharose beads

Dry protein-A-sepharose (SIGMA P3391) was equilibrated in lysis buffer I at 100 mg/ml. After vortexing briefly the beads were spun down at low speed, the supernatant was discarded and the beads were resuspended in 900 µl lysis buffer I. This procedure was repeated twice and the beads were kept in 900 µl lysis buffer I and stored at 4°C.

1. After methanol precipitation, the protein samples were spun at 13,000 rpm and the supernatant was discarded.

2. The samples were left on ice for 5 min and the residual supernatant was removed.

3. The remaining pellet was resuspended in 1 ml of DLPC and sonicated for 1 min at maximum output (until the pellet was completely sonicated into the buffer).

4. 5 µl of preimmune serum (mouse or rabbit, depending on the antibody) were added and the samples were incubated at room temperature for 30 min with constant mixing.

5. 50 µl of protein-A-sepharose beads were added and the samples were incubated for another hour at room temperature with constant mixing.

6. After centrifugation for 2 min at full speed the supenatant was transferred to a new eppendorf and the beads were discarded.

At this point 10 µl of the supernatant were removed for TCA precipitation (7.24).

7. 4µl of 3F4 (Kascsak *et al.*, 1987) (1:500 dilution of mouse anti-hamster PrP monoclonal antibody) or 2µl of 1B3 or 1A8 (Farquhar *et al.*, 1989) (1:1,000 dilution of rabbit anti-PrP polyclonal antibody) were added and the samples were incubated for minimum 1 h (usually overnight) at 4°C.

8. 30 µl of beads were added and the samples were incubated at room temperature with constant mixing for 1 h.

9. The samples were microfuged at low speed and the supernatant was discarded.

10. 500 µl of DLPC were added and vortexed.

11. The beads were spun down at low speed and 500 µl of TN buffer with 1% (w/v) Sarcosyl were added and vortexed.

12. The beads were spun down and the supernatant was discarded.

13. Steps 11 and 12 were repeated twice.

14. 1 ml sterile water was added and votexed.

15. The beads were spun down, the supernatant removed and they were resuspended in 20 µl of sample buffer.

16. The samples were boiled for 10 min and loaded on a 15% acrylamide gel.

For immunoprecipitation of *in vitro* translated PrP (section 7.25) 5-20 µl of sample would be diluted in lysis buffer I (section 7.21.1) and then steps 7-15 were followed, but instead of DLPC and TN buffer, lysis buffer I was used.

7.24 TCA precipitation

solutions

25% (w/v) TCA/2% (w/v) casamino acids (Difco 0230-01)

5% (w/v) TCA

1 N NaOH

1. 10 µl of labeled protein extract was removed (step 6, 7.23) for TCA precipitation. To that 190 µl 1 N NaOH were added and the mixture was incubated at 37°C for 10 min.

2. 1 ml of ice-cold 25% TCA/2% casamino acids was added and incubated on ice for 30 min.

3. The precipitate was collected by filtering under vacuum on Whatman GF/A glass filters. The filters were pre-rinsed in ice-cold 5% TCA.

4. After filtering of the sample the filter was rinsed three times with 3 ml ice-cold 5% TCA and once with 3 ml acetone.

5. The filter was allowed to dry completely at room temperature.

6. The filter was placed in a scintillation vial and covered with 3 ml scintillation fluid (Ultima gold, Packard 6013329) before incorporated radioactivity was measured on the scintillation counter.

7.25 *In vitro* translation

In vitro translation was performed by using the Promega Rabbit Reticulocyte Lysate System (Promega L4960) as per the manufacturer's instructions. L-[^{35}S]-methionine (NEN DuPont NEG 009A) was used to label the newly synthesised proteins and thus enable small quantities of product to be visualized by X-ray fluorography.

1. The RNA was heated to 65°C for 10 min and cooled immediately on ice prior to addition to the reaction, to prevent inhibition by secondary structures.

2. Negative control reactions were performed by adding an equal volume of water instead of the RNA substrate and positive control reactions were performed by using luciferase control RNA provided by the manufacturer.

A standard reaction contained:

17.5 µl rabbit reticulocyte lysate

0.5 µl RNA guard

0.5 µl 1 mM amino acid mixture minus methionine

2 µl 10 µCi/µl L-[^{35}S]-methionine

500 ng *in vitro* produced mRNA substrate in DEPC-treated water (section 7.8)

The volume was made up to 25 µl and the reaction was incubated at 30°C for 1 h.

4. 10 µl of the reaction would be analysed by SDS-PAGE (section 7.21.3).

The GIBCO-BRL Rabbit Reticulocyte Lysate Translation System (GIBCO-BRL 18127-019) was used alternatively to the Promega one in order to compare the efficiency and the background produced. The reaction (final volume 30 µl) was assembled according to the manufacturer's instructions. The control RNA provided encoded bacterial chloramphenicol acetyltransferase.

7.26 Preparation of cell extract using the reporter lysis buffer (for adherent cells grown in tissue culture)

1. 4 volumes of water were added to 1 volume of 5 X reporter lysis buffer to give a 1 X buffer solution.

2. The growth medium was removed from the cells to be assayed. The cells were washed twice with PBS buffer at room temperature, being careful not to dislodge any of the cells. The rest of the final PBS wash was removed with a Pasteur pipette .

3. The cells were lysed by adding a sufficient volume of 1 X reporter lysis buffer to cover the cells (400 µl for a 60 mm culture dish, 900 µl for a 90 mm dish). The dish was rocked slowly several times to ensure complete coverage of the cells and incubated at room temperature for 15 min, rocking the dish halfway through the incubation period.

4. All areas of the plate surface were scraped with a rubber policeman, then the dish was tilted and the cell lysate was scraped to the lower edge of the plate. The cell lysate was transferred to a microcentrifuge tube with a pipet and the samples placed on ice. If several samples were being harvested at the same time, a different rubber policeman was used each time to prevent enzyme carry over from one plate to the next.

5. The lysates were microfuged at full speed in a microcentrifuge for 2 min at 4°C. The supernatant was transferred to a fresh tube.

The extracts would be assayed directly (BioRad protein assay, ß-galactosidase assay, CAT assay) or stored at -20°C.

7.27 ß-galactosidase enzyme assay

This assay is based on that described by Ulman *et al.*, 1967 and in the Hoeffer technical bulletin.

This assay measures the hydrolysis of a ß-galactosidase substrate linked to a fluorogen. Cleavage of 4-methylumbelliferyl-ß-D-galactoside by ß-galactosidase yields the 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, MU) molecule. This molecule excited at 365 nm emits

fluorescence which can be measured at 460 nm wavelength using a fluorometer.

Solutions and buffers

reporter lysis buffer, 5 X (Promega, E3971).
This buffer was diluted 5 times before use.

Reaction cocktail :
25 mM Tris.HCl pH 7.5
125 mM NaCl, 2 mM $MgCl_2$
12 mM ß-mercaptoethanol
0.3 mM 4-methylumbelliferyl-ß-D-galactoside

Add 4-methylumbelliferyl-ß-D-galactoside (Sigma M1633) (20 mg/ml in 100% (v/v) ethanol, stored at -20°C) just before use and vortex immediately to obtain a 0.3 mM concentration.

Glycine-carbonate reagent :
133 mM-glycine
83 mM Na_2CO_3.NaOH pH 10.7
Clarify solution by filtering it through a 0.45 µ cellulose nitrate filter.
This prevents instability in fluorometer readout.

1µM-4-methylumbelliferone (MU) sodium salt (Sigma, M1508)
19.8 mg 4-methylumbelliferone
distilled water to 100 ml
Dilute 10 µl of the above solution into 10 ml distilled water.
Store both solutions at 4°C protected from light.

1. A volume of cellular extract containing 10 µg of protein was transferred to an eppendorf tube. The volume was made up to 40 µl with 1 X lysis buffer.

2. 160 ml of the reaction cocktail was added, mixed and incubated 37°C for 30 min.

3. The reaction was stopped by adding 50 µl of 0.2 M Na_2CO_3 and cooling on ice.

4. The solution was clarified by centrifugation in a microcentrifuge for 1-2 min.

5. The fluorescence (resulting of MU yield) was read by using the Hoeffer fluorometer TK100, following the procedure described below:

The fluorometer was calibrated with a MU solution, as explained:

1. The fluorometer was switched on for at least 15 min before use.

The SCALE knob of the fluorimeter was adjusted to 50% sensitivity (5 complete turns from the fully anticlockwise position).

2. A glass cuvette was filled with 2.0 ml of glycine-carbonate reagent, placed in the fluorometer and the display was brought to zero by adjusting the ZERO knob of the fluorometer.

3. A glass cuvette was filled with 1.9 ml of glycine-carbonate reagent plus 100 µl of a 1 µM/MU solution (gives 2 ml of glycine-carbonate solution containing 0.1 nmole of MU) and the SCALE knob was adjusted to obtain a readout of 500. So 500 fluorescence corresponds to 0.1 nmole MU and one unit of ß-galactosidase corresponds arbritarily to enzyme level yielding 0.1 nmole MU in 30 min at 37°C.

After the calibration the fluorescence of samples (cellular extract) could be read:

100 µl of the supernatant was used and the volume was made up to 2.0 ml with glycine-carbonate reagent. If MU level in 100 µl was too high to be measured, the measurement would be done on 10 or 1 µl of supernatant (volume of supernatant used for assay depends on ß-galactosidase level in cellular extract, and then of quantity of MU yield).

To check the right linearity of the assay, the fluorescence of samples containing known quantities of E. coli ß-galactosidase enzyme (Boehringer 567799), would be measured, (ß-galactosidase 0 to 200 pg in 40 µl 1 X-lysis buffer treated as explained above for samples).

7.28 CAT assay

This method is based on that described by Seed and Sheen, 1988.

Chloramphenicol acetyltransferase (CAT), encoded by a bacterial drug-resistance gene, inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups. This gene is not found in eukaryotes, and therefore eukaryotic cells contain no background level of CAT activity.

This assay is based on liquid scintillation counting of CAT reaction products. Cell extracts were incubated in a reaction mix containing [^{3}H]-labeled acetyl CoA and chloramphenicol. CAT enzyme, present in cellular extract, catalyzes transfer of the acetyl group from acetyl CoA to chloramphenicol. This assay does not require any extraction because [^{3}H] acetyl CAT (but not [^{3}H] acetyl CoA) is soluble in the organic phase containing scintillant; liquid scintillation counting detects the product but not the substrate. A continuous data stream can be generated, providing quantitative results within minutes or several hours.

Solutions and buffers

reporter lysis buffer, 5 X.

1 M Tris.HCl pH 7.8

5 mM Chloramphenicol

1. Cell extract containing 25 mg protein and 100 mM Tris.HCl pH 7.8 were mixed in a 5 ml plastic mini-scintillation vial to give a total volume of 50 ml.

2. To the 50 ml of cell extract 200 ml of the following freshly prepared reaction mix were added:

1 ml [^{3}H] acetyl CoA (250 Ci/ml from Amersham, code TRK. 688)
50 ml 5 mM chloramphenicol
25 ml 1 M Tris.HCl pH 7.8
124 ml H_2O per reaction

3. The reaction mixture was gently overlaid with 3 ml of water-immiscible scintillation fluid (Econofluor-2, NEN No. NEF 969), and the vials were placed directly in the scintillation counter to be counted for 1 min. Vials were counted repeatedly to give a time course curve (arrange to cycle vials).

4. The linearity of the reaction was checked with known E. coli CAT concentrations from 0 to 0.0100 u/50 µl Tris.HCl pH 7.8 (E. coli CAT, Pharmacia 27-0847-01).

7.29 Tissue Culture

7.29.1 Solutions

Cell Culture Medium

distilled water	170 ml
10 X Dulbecco's modified Eagle's medium (DMEM, Gibco)	20 ml
sodium bicarbonate (7.5% (w/v) stock, Gibco)	6.6 ml
glutamine, 200 mM + pyruvate, 100 mM (Gibco)	4 ml
ß-mercaptoethanol (Sigma)	200 ml
foetal calf serum (Gibco)	20 ml

Trypsin

0.25% trypsin (from 2.5% (w/v) solution in normal saline, Gibco)
1 mM EDTA (AnalaR, BDH)
1% (v/v) chick serum (Gibco)
Store at -20°C.

Nerve growth factor (NGF) (SIGMA, N0513)
Hygromycin B (50 mg/ml Boehringer Mannheim, 843555)

7.29.2 General notes

Tissue culture cells were maintained in the Centre for Genome research, initially obtained from 'European Collection of Animal Cell Cultures', or the 'American Type Culture Collection.

All tissue culture manipulations were undertaken inside a laminar flow sterile hood (ICN, Flow). All objects were washed with 70% (v/v) industrial methylated spirits (IMS, BDH) before being placed in the hood. Cells were maintained in tissue culture grade plastic flasks and culture dishes (Corning). The cells were grown in a humidified incubator at 37°C, in a 6% CO_2 atmosphere. The growth medium used was DMEM supplemented with 10% (v/v) foetal calf serum. Medium in the flasks was replaced when it began to turn from orange to yellow, usually every 2 days. Confluent cultures were passaged into fresh flasks at 1/10 their original density. The medium was removed from the flasks the cells were then rinsed twice with PBS pre-warmed to 37°C, then overlaid with 1 ml trypsin and incubated at 37°C until the cells lifted from the surface of the flask. The flasks were tapped to remove the cells from the base. The trypsin was neutralised by the addition of 5ml of fresh medium and a single cell suspension formed by drawing the medium several times through a glass pipette. The cells were pelleted by centrifugation at 1,200 rpm for 5 min, the medium removed and the pellet resuspended in 5ml of fresh culture medium. 0.5 ml of cell suspension was then added to pre-warmed fresh culture medium, and placed in a new flask.

7.29.3 Freezing Cells

Cells were removed from the growth surface by trypsin digestion and resuspended in culture medium to which 1/10 volume of DMSO (AnalaR, BDH) was then added. The cell suspension was then centrifuged at 1,200rpm for 5 min, the medium was removed, and the pellet resuspended in freezing medium (culture medium supplemented with 10% (w/v) DMSO). 0.5ml of freezing medium was used for each 5 X 10^6 cells. The suspension was then placed into cryotubes (Nunc) in 0.5 ml aliquots and frozen at -80°C. After 24 h, the vials were transferred to a liquid nitrogen cell bank (XLC110, Minnesota Valley Engineering Cryogenics).

7.29.4 Thawing cells

Vials of frozen cells were removed from the liquid nitrogen storage bank and thawed rapidly. The cell suspension was then transferred to a 15 ml centrifuge tube (Corning) containing 4.5 ml of culture medium using a sterile Pasteur pipette. The cells were pelleted by centrifugation at 1,200rpm for 5 min, resuspended in 10 ml of fresh culture medium and transferred to a gelatinised 25 cm^2 flask. The medium was changed when the cells were firmly attached to the growth surface (usually after about 7 h or next day). The cells were subsequently maintained as above.

7.29.5 Calcium phosphate transfection of cells

Solutions

BES solution
1.630 g NaCl
1.060 g BES (Calbiochem 391334)
0.020 g Na_2HPO_4
Water was added to 100 ml the pH was adjusted to 6.95 and the solution
was filter sterilised and stored at 4°C in 20 ml aliquots.

1. Cells were grown to 40-50% confluence and the medium was changed 1 h (at least) before transfection. Usually a 25 cm^2 flask or 90 mm dish were used and 4.5 ml of medium. The cells were placed in an incubator at 2.5% CO_2 and 37°C.

2. The transfection mix was prepared as following:

10 µl of 1 µg/µl plasmid DNA
25 µl $CaCl_2$ 2.5 M
215 µl sterile water (filtered AnalaR)
and was mixed well

For stable transfections the DNA was linearised by restriction digest with an appropriate enzyme and purified through a "Wizard" column (section 7.6.3) before adding to the transfection mix.

For cotransfections a 5:1 ratio of DNA:control DNA (usually pCAT plasmid) was used.

3. 250 µl of BES were added dropwise to the transfection mix vortexed and left for 15 min at room temperature.

4. The transfection solution was added dropwise to the cells. At this point the medium should look pink with obvious fine grains of DNA precipitate.

5. The cells were incubated overnight in a 2.5% CO_2, 37°C incubator, the medium was replaced the next day and the cells returned to a 6% CO_2, 37°C incubator.

6. The cells were left for 2-3 days before any further treatment.

7.30 *In situ* staining of cells for ß-galactosidase

Solutions

0.1 M sodium phosphate, pH 7.0
60 mM Na_2HPO_4
40 mM NaP_2HO_4

fix solution
1% (v/v) glutaradehyde in 0.1 M sodium phosphate pH 7.0 and 1 mM $MgCl_2$

X-gal solution
0.2% (w/v) X-gal (Bachem SRB020)
in 10 mM sodium phosphate pH 7.00
1 mM $MgCl_2$
150 mM NaCl
3.3 mM $K_4Fe(CN)_6 \cdot 3H_2O$
3.3 mM $K_3Fe(CN)_6$

The solution was prepared freshly each time and filtered through a 0.2 micron syringe filter to remove large crystals present.

Cells transfected with ß-galactosidase containing plasmids were tested for ß-galactosidase enzyme expression by incubating with the substrate X-gal (5-bromo-3-indolyl-ß-D-galactosidase). Transfected cells could be visualized under the microscope as the would appear blue after the incubation.

1. The cells were washed 5 times with PBS.
2. The cells were fixed with 1% (v/v) glutaraldehyde solution for 15 min.
3. The glutaraldehyde solution was removed, the X-gal solution was added and incubated for at least 2 h overnight at 37°C.
4. The X-gal solution was removed, the cells were covered with 70% (v/v) glycerol and viewed with a light microscope.

7.31 Transgenics

7.31.1 Animal maintenance

Mice were bred under standard controlled temperature (22°C) and lighting (12 h light, 12 h dark). Mice were fed with mouse chow (SDS Ltd, UK) and water. At 21 days of age offspring were weaned from their mothers, separated according to sex and ear-punched for identification. All manipulations on live animals were carried out according to UK Home Office Regulations.

7.31.2 Generation of transgenic animals

DNA fragments produced by BssHII restriction digestion from the plasmid constructs (see Figure 2.3.6) were isolated by gel electrophoresis. The fragments were excised from the gel and the DNA was extracted (see section 7.6.2) and purified (see section 7.6.3). The DNA was ethanol precipitated (7.6.4), resuspended in sterile, particle free water to give 100

ng/µl. An aliquot of this was electrophoresed in parallel with known DNA standards so that the concentration could be estimated precisely. DNA was diluted 1:10 (final concentration 10 ng/µl) in particle free water and used for microinjection into pronuclei of fertilized mouse eggs (C57BL/6 X CBA/Ca) by Dr P.Estibeiro and Rosie Allen. The eggs were transferred to the oviducts of pseudopregnant foster mothers according to methods described in (Hogan *et al.*, 1986). Weanling animals were screened for the presence of HaPrP gene by dot-blot or Southern analysis of tail, or ear biopsy. Founder mice were mated to F1(C57BL/6 X CBA/Ca) mice to yield the first generation of transgenic animals. First generation transgenic animals were mated to F1 mice as before to give second generation transgenic animals. Non-transgenic litermates were incorporated in the experiments.

7.31.3 Calculation of transgene copy number

1 copy of the NSE HaPrP fragment, which is 4 kb, would correspond to 6.6 pg of the fragment per 10 µg of genomic DNA ($2x10^9$ bp per diploid genome) and 1 copy of the TH HaPrP fragment (6.5 kb), would correspond to 10.8 pg of that fragment per 10 µg of genomic DNA. Different amounts of these fragments (representing 1, 2, 5, or 10 copies) were mixed with 10 µg of genomic DNA, digested with the same restriction enzyme as the rest of the genomic DNA samples and used as a copy number control for the dot blots.

Southern blots hybridised with the random primed HaPrP ORF fragment, which is 90% homologous to the MoPrP ORF, would reveal the endogenous, single copy, MoPrP gene, which could serve as a copy number control.

7.32 *In situ* hybridisation

Frozen mouse brains were sectioned on a cryostat (Anglia Scientific) to give 10 µ sections and paraffin embedded mouse brains were sectioned on a microtome (Anglia Scientific) to give 6-8 µ sections (either coronal or horizontal).

7.32.1 Paraffin embedding of mouse brains

solutions

saline
0.8% (w/v) NaCl in DEPC-treated water

Mouse brains were dissected in five segments (coronal) so that they could be effectively fixed in 4% paraformaldehyde over night. The segments were immersed in the following solutions:

PBS for 30 min
saline: 0.8% (w/v) NaCl in DEPC-treated water
for 30 min
saline:ethanol 1:1 for 15 min
dehydration through a series of ethanol solutions (50%-100% v/v) in water for 10 min each
100% ethanol 2 X 10 min
histoclear (National Diagnostics) for 2 X 30 min then 1 X 1 h
paraffin (Shandon), 56°C 2 X 60 min

The brain segments were embedded in blocks of paraffin and stored in 4°C.

7.32.2 Pretreatment of glass slides

Glass slides (Chance Propper) were cleaned by a brief immersion in 10% (v/v) HCl, followed by a 70% ethanol wash and rinsed in water then 95% ethanol. They were heated to 150°C and allowed to cool before they were immersed in 4% 3-aminopropyltrioxysilane (TESPA) (v/v) (Sigma A3648) and then acetone. They were rinsed twice in acetone, then water and allowed to dry at 42°C. Following this treatment they could be stored dessicated in the fridge for up to a month.

7.32.3 Preparation of sections for *in situ* hybridisation

solutions

Paraformaldehyde

PBS was heated to 65°C on a stirrer, then the paraformaldehyde (Sigma P-6148) was added to 4% (w/v).

5 M NaOH was added to help the paraformaldehyde to dissolve (approximately 5 drops).

Once in solution, the solution was cooled to room temperature and adjusted to pH 7.4 with concentrated HCl.

saline

0.8% (w/v) NaCl

acetic anhydride

0.25% (v/v) was added to 0.1 M triethanolamine in 0.8% (w/v)NaCl.

(Acetic Anhydride is very unstable and was made as close to usage time as possible).

All manipulations were carried out at room temperature. All solutions were 0.1% (v/v) DEPC-treated and autoclaved. Microscope slides were placed back-to-back in Coplin jars, with the following solutions poured on or off for each step. For each step, 30-35 ml of each solution per Coplin jar was required.

7.32.3a from frozen brain sections

1. Sections were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min.
2. Sections were rinsed in two changes of PBS for 5 min.
3. Sections were proteinase K treated by immersing in 20 mg/ml proteinase K in 50 mM Tris.HCl (7.4), 5 mM EDTA for 5 min.
4. Sections were rinsed in PBS for 5 min.

5. Sections were fixed again in 4% (w/v) paraformaldehyde in PBS for 10 min. (The same solution was reused from step 1).

6. Sections were rinsed in water for 5 min.

7. The sections were acetylated by placing in 0.25% (v/v) acetic anhydride for 2 X 10 min.

8. The sections were rinsed briefly in PBS.

9. The sections were rinsed in saline for 5 min.

10. The sections were dehydrated by passing through a series of ethanols (30 %-100%) for 1 min each and then 2 X 5 min in 99% ethanol.

11. Sections were immersed in chloroform for 5 min, rinsed briefly in two changes of absolute ethanol and a final rinse in 95% ethanol.

12. The slides were removed from the Coplin jars and allowed to air dry before hybridisation.

7.32.3b from paraffin embedded brain sections

Sections were immersed in xylene 2 X 10 min then 99% ethanol 2 X 2 min and rehydrated through a series of ethanols (99%-50%) 1 min each, then ethanol:saline 1:1 for 2 min, then saline for 5 min, PBS for 5 min and then the procedure described above (7.32.1a) was followed.

7.32.4 Hybridisation Conditions

7.32.4a for oligonucleotide probes

hybridisation buffer
4 X SSC (section 7.1)
50% (v/v) deionised formamide
10% (w/v) dextran sulphate
1 X Denhardt's solution (section 7.1)
0.1% (w/v) SDS
500 µg/ml salmon sperm DNA
250 µg/ml yeast tRNA
the buffer was de-gassed before use

Oligonucleotides probes were labeled by homopolymer tailing using [α-^{35}S]-dATP and terminal transferase as described (see section 7.17.3)

1. The prepared ^{35}S-tailed probe (resuspended in 10 mM DTT in TE) was diluted to 2 x 10^6 cpm/ml in hybridisation buffer. DTT is also added to this mixture to a final concentration of 50 mM.

2. 100 ml of the probe mixture was carefully layered on to each microscope slide. A piece of parafilm cut to the size of the microscope slide was then layered over the probe mixture, allowing the probe and hybridisation mixture to cover all the sections. Air bubbles under the parafilm were avoided.

3. The slides were placed in a humidified container, sealed, and incubated at 37°C overnight.

4. After hybridisation, the parafilm was carefully removed using forceps.

5. The slides were placed back in Coplin jars, and the hybridised sections washed in four changes of 1 X SSC for 15 min at 55°C or 60°C, and then two changes of 1 X SSC for 30 min at room temperature.

6. The slides were rinsed briefly in dH_2O, then left to air dry.

7.32.4b for riboprobes

hybridisation buffer
50% (v/v) deionised formamide
10% (w/v) dextran sulphate
1 X Denhardt's solution
300 mM NaCl
20 mM M Tris.HCl pH 8.0
10 mM sodium phosphate pH 8.0
100 mM EDTA
1 mg/ml yeast tRNA
sterile DEPC-treated water to 20 ml
the buffer was de-gassed before use

TNE
10 mM Tris.HCl pH 7.5
0.5 M NaCl
5 mM EDTA

1. Riboprobes were synthesised as described in section 7.17.4. Riboprobes resuspended in appropriate volume of TE/50 mM DTT to give approximately 1 X 10^6 cpm/ml were finally diluted 1:10 in hybridisation buffer/50 mM DTT.

2. 100 ml of the probe mixture was carefully layered on to each microscope slide. A piece of parafilm cut to the size of the microscope slide was then layered over the probe mixture, allowing the probe and hybridisation mixture to cover all the sections. Air bubbles under the parafilm were avoided.

3. The slides were placed in a humidified container, sealed, and incubated at 55°C overnight.

4. After hybridisation, the parafilm was carefully removed using forceps.

5. The slides were placed back in Coplin jars, and the hybridised sections washed in the following solutions:

5 X SSC/10 mM DTT for 30 min, 55°C
50% formamide/2 X SSC/10 mM DTT for 20 min, 65°C
TNE for 3 X 10 min, 37°C
TNE with 40 mg/ml RNase for 30 min, 37°C
50% formamide/2 X SSC/10 mM DTT for 20 min, 65°C
2 X SSC for 3 X 10 min, room temperature
0.1 X SSC for 3 X 10 min, room temperature
30% ethanol in 0.3 M NH_4 acetate for 1 min, room temperature
50% ethanol in 0.3 M NH_4 acetate for 1 min, room temperature
70% ethanol in 0.3 M NH_4 acetate for 1 min, room temperature
94% ethanol in 0.3 M NH_4 acetate for 1 min, room temperature
99% ethanol in 0.3 M NH_4 acetate for 1 min, room temperature
100% ethanol 2 X 5 min

6. The slides were rinsed briefly in dH_2O, then left to air dry.

7.32.5 Autoradiography

Detection of hybridisation signals was by autoradiography. Slides were placed against Kodak XAR-5 film for 2-7 days for a low resolution image. For a high resolution image, a photographic liquid emulsion (LM-1, Amersham RPN.40) was used. All manipulations were carried out under safelight conditions.

1. The emulsion was melted by placing in a 43°C water bath.
2. Once melted, the emulsion was poured into a dipping vessel.
3. Blank microscope slides were first dipped into the emulsion until no bubbles could be seen on the slides.
4. Each test slide was dipped evenly into the emulsion vertically for 1-2 sec.
5. Slides were withdrawn from the emulsion, allowed to drain briefly then the back of each wiped with a tissue to remove excess emulsion.
6. The slides were laid flat to dry.
7. Once dry, the slides were laid flat in a container containing silica gel, and left at room temperature overnight.
8. The slides were transferred into a plastic slide box containing more silica gel, and sealed with tape. The box was covered with two layers of aluminium foil and stored at 4°C for exposure.
9. When ready for developing, the emulsion-dipped slides were brought back to room temperature.
10. Hybridised sections were placed in D19 developer (Kodak, 502 7065) for 2 min, then transferred to 1% (v/v) acetic acid, 1% (v/v) glycerol for 1 minute, then to 30% (w/v) sodium thiosulphate for 2 min.
11. The sections were finally washed in a large volume of running water for 10 min.

7.32.6 Counterstaining and Mounting of Sections

1. While the sections were still wet, the slides were placed in 0.1% (w/v) methyl green stain (Sigma, M-8884), and stained for 1-2 min.
2. The sections were briefly rinsed in water to remove excess stain and left to air dry.

3. Sections were mounted in DePeX (BDH, 36125), and covered with a glass coverslip.

APPENDIX 1

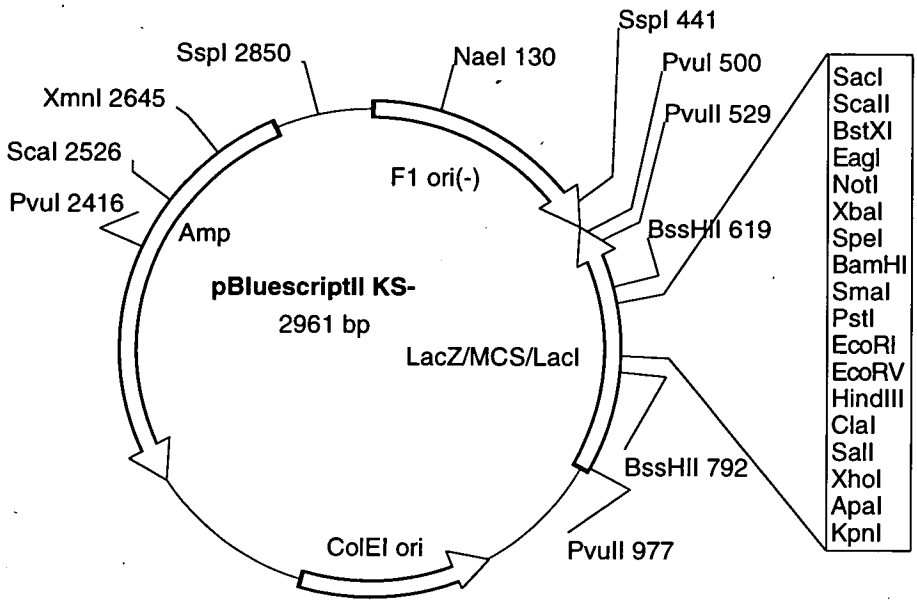
Synthesised oligonucleotides

name	oligonucleotide sequence
569G	ACATGCTTCATGTTGG
241V	CTGCTTGATGGTGATGTTGACAGCAGTC
325S	TGGAACAAGCTGAGTAAGC
324S	GAACTTCACCAAGACCGA
164N	ACCAGGATCCAGGAAGACCTTCCTCATCCCAC
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGGC
5'HaP	GT AAGCTT TGTGGCTATGTG
3'HaP	GC GAATTC TGGTCTACTGTAC
SV40.1	GA CTCGAG ATCATAATCAGCCATA
SV40.2	GG GGTACC AGACATGATAAG
HNE I	AGCTTGCGGCCGCG
HNE II	AATTCGCGGCCGCA
EcoRI l.	CGGAATTCCG
HindIII l.	CCAAGCTTGG
Mo-a	GAGGAGAAAAGCACGGTGCTGCTGGATCTT
	CTCCCGTCGTAATAG
Mo-s	CTATTACGACGGGAGAAGATCCAGCAGCAC
	CGTGCTTTTCTCCTC
Ha-a	GAGGAGAACAGCACCGCGCTGGACCTTCTTC
	CATCGTAGTAG
Ha-s	CTACTACGATGGAAGGTCCAGCGCGGTGCTG
	TTCTCCTC
SV40-a	TAAAGCAAGTAAAACCTCTACAAATGTGGT
	ATGGCTGATTATGAT
SV40-s	ATCATAATCAGCCATACCACATTTGTAGAGG
	TTTTACTTGCTTTA
TH-a	TTGGGGACGTGACAGCCTCGGCCTGCTTGGCA
	TCCTGCTCTGAGA
lacZ.1	TCGGCG GAATTC CAGCTG
lacZ.2	AAT GCGGCCGC CGAGTTTGTCA

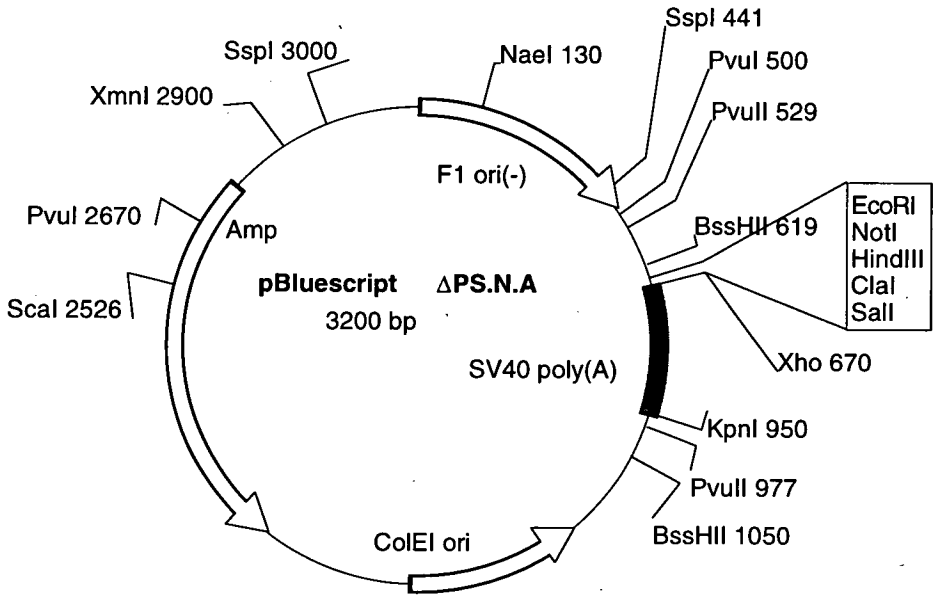
3'UTR.1	TCAG CTCGAG GAAGCCTCCCTGCTTGTA
3'UTR.2	GTC GGATCC AAACATTTATAAACTTTTA
5'ex.2.a	CAGT GTCGAC ATCAGCCATCATGGCGAACC
5'ex.1.a	CAGT GTCGAC CGCGGCGTCCGAGCAGCAGA
5'Ko	CAGT GTCGAC GCCGCCACCATGGCGAACCT
3'HalacZ	CAGT TCGCGA GCTCCCACCATCAGGAAGATGAG
5'ex.2.b	ACTG CTCGAG CTTTGTGGCTATGTGGA
5'ex.1.b	CAGT CTCGAG CGGCGTCCGAGC
3'UTR.3	GC GGATCC TGGTCTACTGTAC
Hind.ad.1	AGCTTGGGCGTCA
Hind.ad.2	ACCCGCAGT
APPf	GTGAAGACAAAGTAGTAGAAG
APPr	ATTTCCAGAAAGCCAAAGAGA

APPENDIX 2

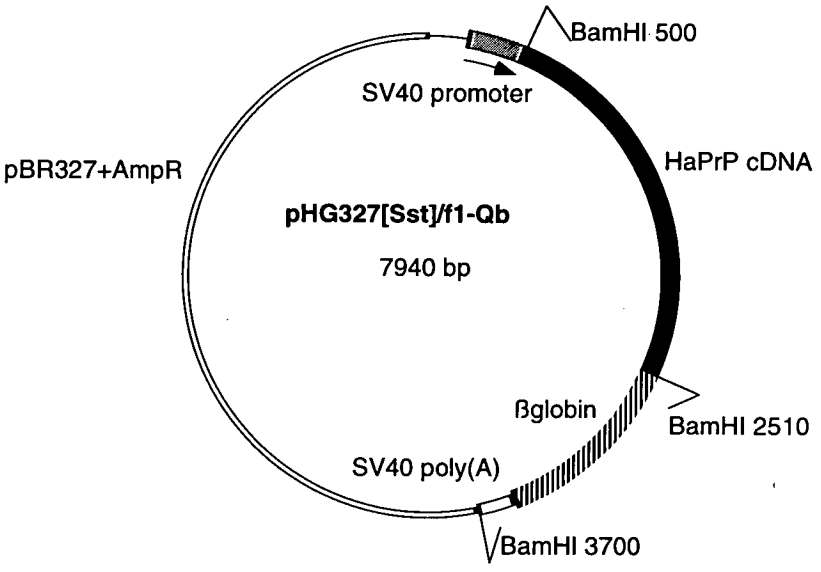
Plasmid maps



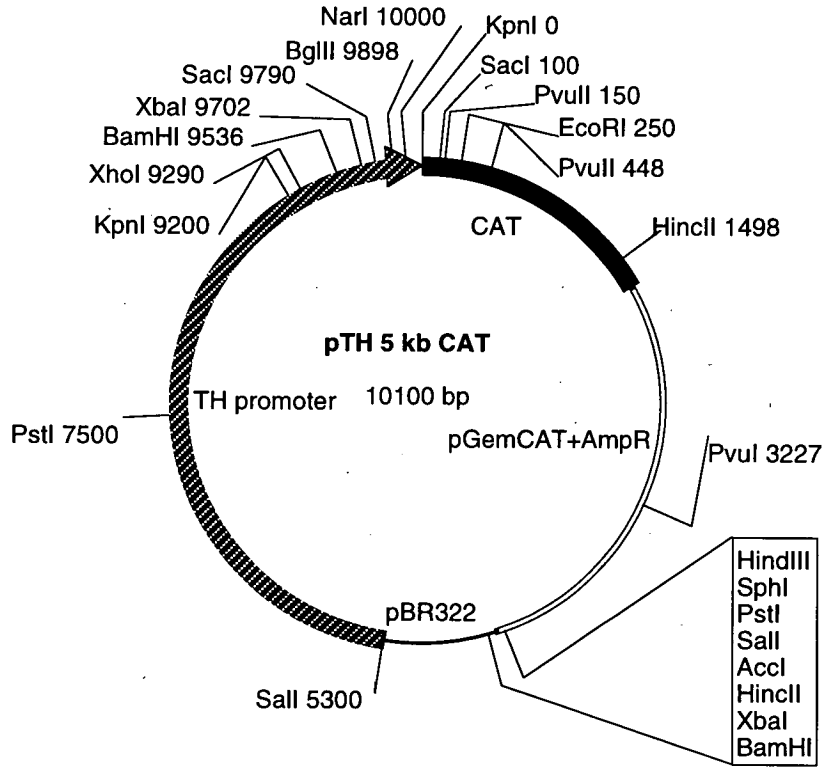
pBluescriptII KS- (Stratagene)



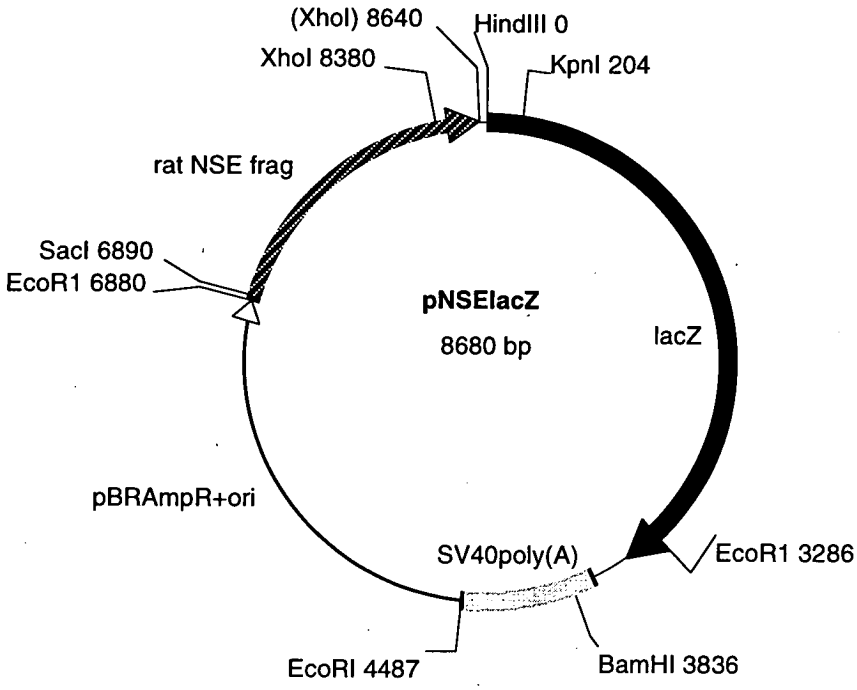
pBluescript ΔPS.N.A. (constructed by A.Voulgari)



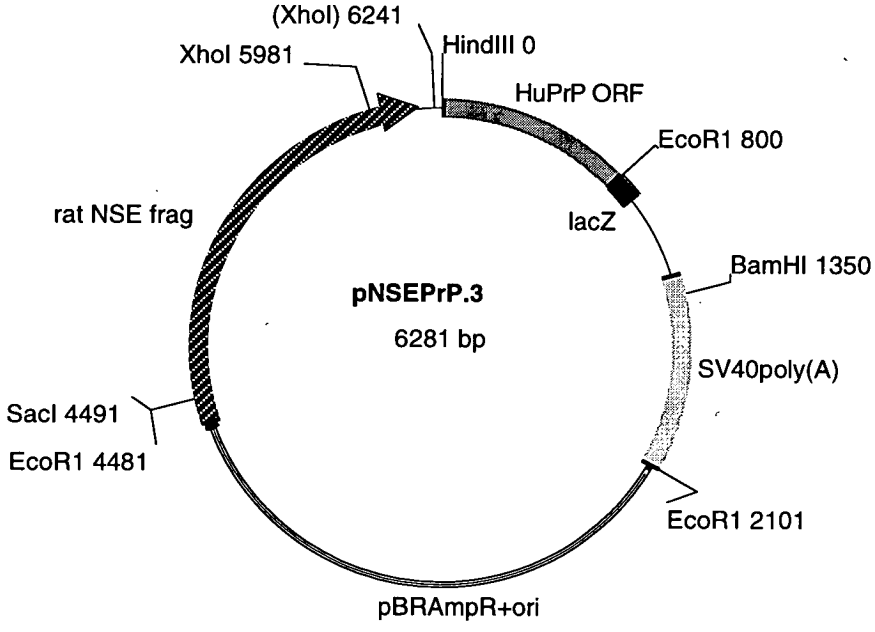
pHG327[Sst]/f1-Qb (Dr N. Robakis, unpublished plasmid)



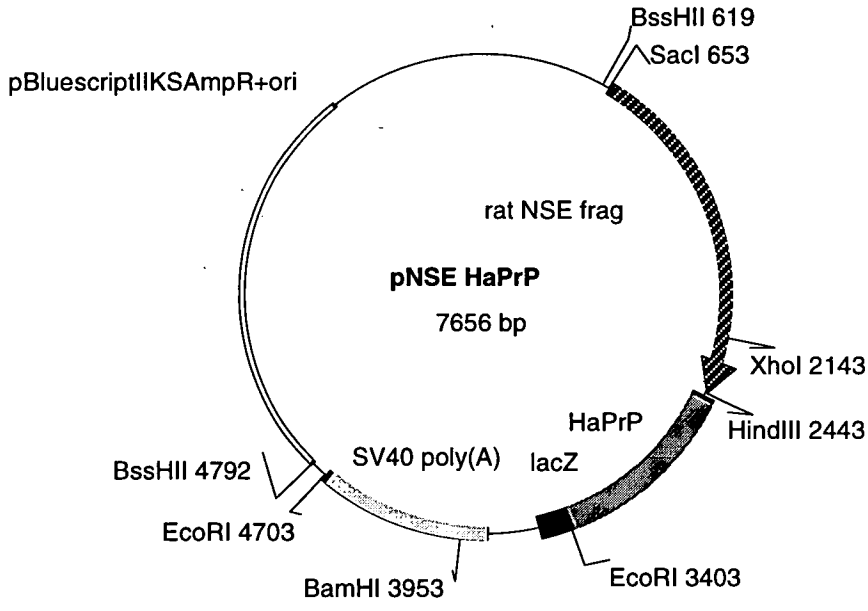
pTH 5 kb CAT (Dr J. Mallet, unpublished plasmid)



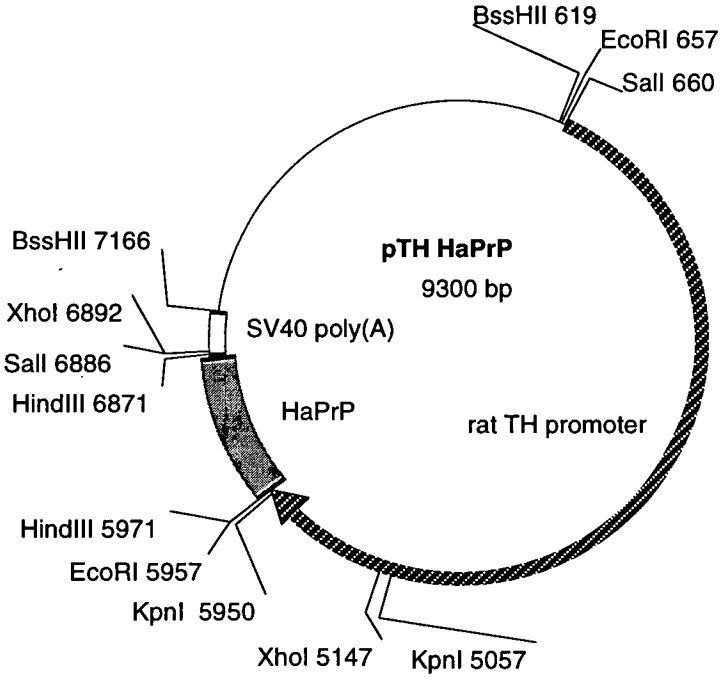
pNSElacZ (Fross-Petter *et al* 1990)



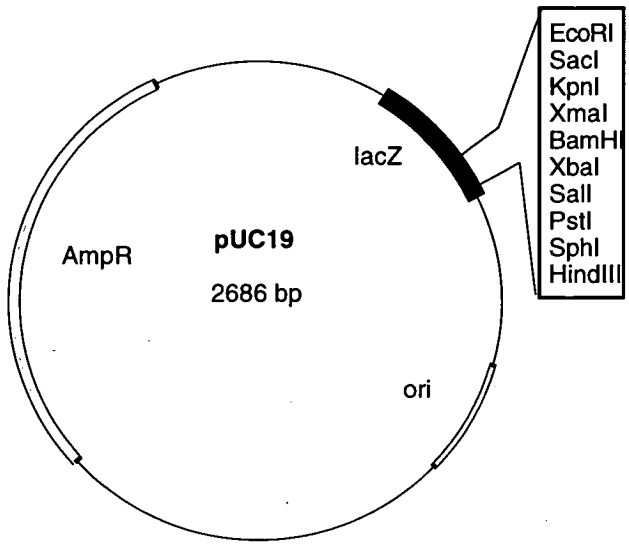
pNSEPrP.3 (constructed by Dr P. Estibeiro)



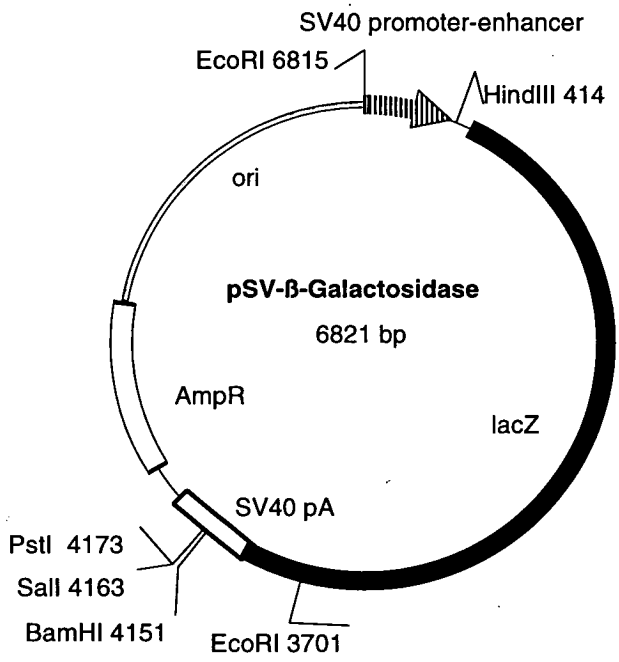
pNSE HaPrP (constructed by A.Voulgari)



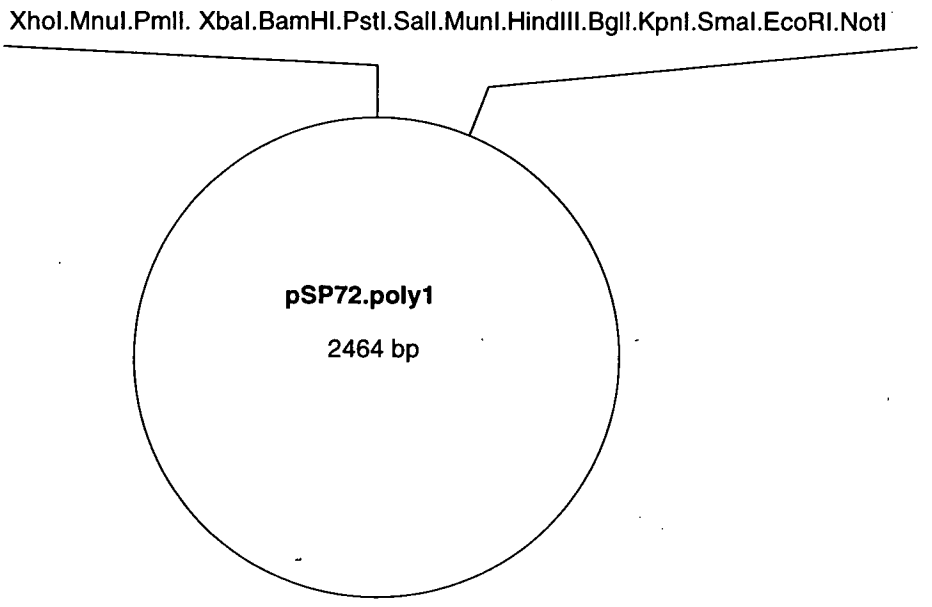
pTH HaPrP (constructed by A.Voulgari)



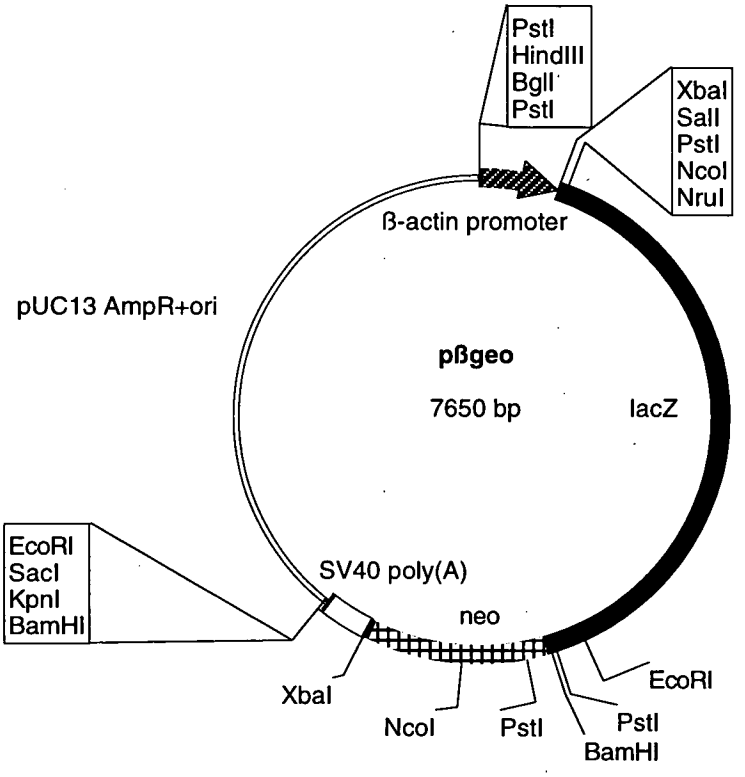
pUC19 (New England Biolabs)



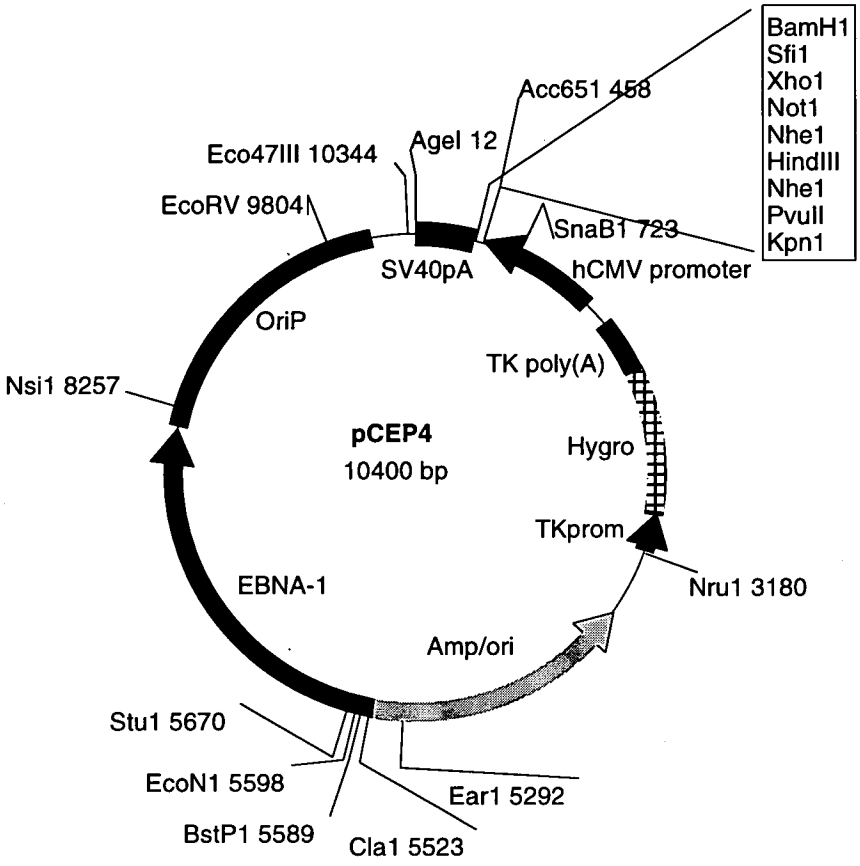
pSV-ß-Galactosidase (Promega)



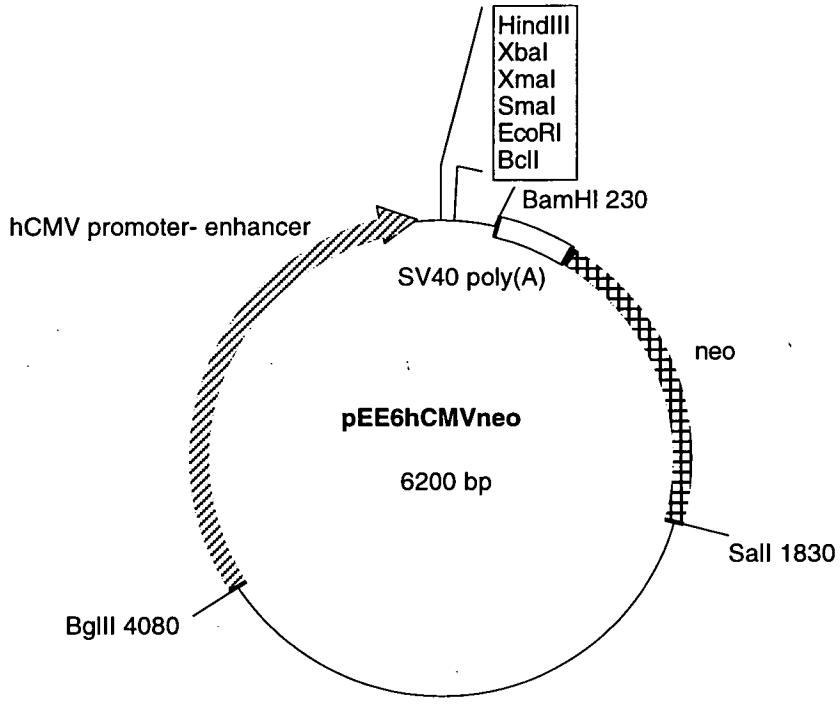
pSP72.poly1 (constructed by Dr S. Morley)



pßgeo (constructed by Dr W. Skarnes)



pCEP4 (Invitrogen)



pEE6hCMVneo (Celltech)

pEE6hCMVneoHaPrP: the HaPrPORF (with a short 5' UTR sequence comprising of the 'optimal' initiation of translation sequence was cloned into the HindIII-EcoRI sites of the polylinker

BIBLIOGRAPHY

1992. Rapid recovery of DNA from agarose gels. *Trends in genetics* 8:81.

Aguzzi, A., S. Brandner, U. Sure, D. Ruedi, and S. Isemann. 1994. Transgenic and knock-out mice:models of neurological disease. *Brain Pathology* 4:3-20.

Aiken, J. M. and R. F. Marsh. 1990. The search for scrapie agent nucleic acid. *Microbiological Reviews* 54:242-246.

Aiken, J. M., J. L. Williamson, L. M. Borchardt, and R. F. Marsh. 1990. Presence of mitochondrial D-loop DNA in scrapie-infected brain preparations enriched for prion protein. *Journal of Virology* 64:3265-3268.

Akowitz, A., T. K. Sklaviadis, E. E. Manuelidis, and L. Manuelidis. 1990. Nuclease resistant polyadenylated RNAs of significant size are detected by PCR in highly purified Creutzfeldt-Jakob disease preparations. *Microbial Pathogenesis* 9:33-45.

Akowitz, A., E. E. Manuelidis, and L. Manuelidis. 1993. Protected endogenous retroviral sequences copurify with infectivity in experimental Creutzfeldt-Jakob disease. *Archives of Virology* 130:301-316.

Alper, T., D. A. Haig, and M. C. Clarke. 1966. The exceptionally small size of the scrapie agent. *Biochemical and Biophysical Research Communications* 22:278-284.

Alper, T., W. A. Cramp, D. A. Haig, and M. C. Clarke. 1967. Does the agent of scrapie replicate without nucleic acid? *Nature* 214:764-766.

Baker, H. F., R. M. Ridley, and G. A. H. Wells. 1993. Experimental transmission of BSE and scrapie to the common marmoset. *Veterinary record* 132:403-406.

Baldwin, M. A., A. L. Burlingame, and S. B. Prusiner. 1993. Mass spectrometric analysis of a GPI-anchored protein: the scrapie prion protein. *Trends in Analytical Chemistry* 12:239-248.

Banerjee, S. A., P. Hoppe, M. Brilliant, and D. M. Chikaraishi. 1992. 5' flanking sequences of the rat tyrosine hydroxylase gene target accurate tissue-specific, developmental, and transsynaptic expression in transgenic mice. *Journal of Neuroscience* 12:4460-4467.

Barlow, R. M. and D. J. Middleton. 1990. Dietary transmission of bovine spongiform encephalopathy to mice. *Veterinary record* 126:111-112.

Barry, R. A., S. B. H. Kent, M. P. McKinley, and et al. 1986. Scrapie and cellular prion protein share polypeptide epitopes. *Journal of Infectious Diseases* 153:848-854.

Basler, K., B. Oesch, M. Scott, and et. 1986. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell* 46:417-428.

Bazan, J. F., R. J. Fletterick, M. P. McKinley, and S. B. Prusiner. 1987. Predicted secondary structure and membrane topology of the scrapie prion protein. *Protein Engineering* 1:125-135.

Bell, J. E. and J. W. Ironside. 1993. Neuropathology of spongiform encephalopathies in humans. *British Medical Bulletin* 49:738-777.

BellingerKawahara, C., J. E. Cleaver, T. O. Diener, and S. B. Prusiner. 1987a. Purified scrapie prions resist inactivation by UV irradiation. *Journal of Virology* 61:159-166.

BellingerKawahara, C., T. O. Diener, M. P. McKinley, D. F. Groth, G. Smith, and S. B. Prusiner. 1987b. Purified scrapie prions resist inactivation by procedures that hydrolyse, modify, or shear nucleic acids. *Virology* 160:271-274.

Bendheim, P. E., A. Potempska, R. J. Kascsak, and D. C. Bolton. 1988. Purification and partial characterization of the normal cellular homologue of the scrapie agent protein. *Journal of Infectious Diseases* 158:1198-1208.

Bendheim, P. E., H. R. Brown, R. D. Rudelli, L. J. Scala, N. L. Goller, G. Y. Wen, R. J. Kascsak, N. R. Cashman, and D. C. Bolton. 1992. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology* 42:149-156.

Bertoni, J. M., P. Brown, L. G. Goldfarb, R. Rubenstein, and D. C. Gajdusek. 1992. Familial Creutzfeldt-Jakob disease (codon 200 mutation) with supranuclear palsy. *Journal of the American Medical Association* 268:2413-2415.

Bessen, R. A., D. A. Kocisko, G. J. Raymond, S. Nandan, P. T. Lansbury, and B. Caughey. 1995. Non-genetic propagation of strain-specific properties of scrapie prion protein. *Nature* 375:698-700.

Bessen, R. A. and R. F. Marsh. 1992. Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *Journal of Virology* 66:2096-2101.

Birnboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* 7:1513.

Bolton, D. C., M. P. McKinley, and S. B. Prusiner. 1982. Identification of a protein that purifies with the scrapie agent. *Science* 218:1309-1311.

Bolton, D. C., R. K. Meyer, and S. B. Prusiner. 1985. Scrapie PrP 27-30 is a sialoglycoprotein. *Journal of Virology* 53:596-606.

Borchelt, D. R., A. Taraboulos, and S. B. Prusiner. 1992. Evidence for synthesis of scrapie prion proteins in the endocytic pathway. *Journal of Biological Chemistry* 267:16188-16199.

Borchelt, D. R., M. Rogers, N. Stahl, G. C. Telling, and S. B. Prusiner. 1993. Release of the cellular prion protein from cultured cells after loss of its glycoinositol phospholipid anchor. *Glycobiology* 3:319-329.

Bradley, R. and J. W. Wilesmith. 1993. Epidemiology and control of bovine spongiform encephalopathy (BSE). *British Medical Bulletin* 49:932-959.

Brinster, R. L., J. M. Allen, R. R. Behringer, R. E. Gelinas, and R. D. Palmiter. 1988. Introns increase transcriptional efficiency in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 85:836-840.

Brown, H. R., N. L. Goller, R. D. Rudelli, G. S. Merz, G. C. Wolfe, H. M. Wisniewski, and N. K. Robakis. 1990. The mRNA encoding the scrapie agent protein is present in a variety of non-neuronal cells. *Acta Neuropathologica* 80:1-6.

Brown, P., F. Cathala, R. F. Raubertas, D. C. Gajdusek, and P. Castaigne. 1987. The epidimiology of Creutzfeldt-Jakob disease: conclusion of a 15-year investigation in France and review of the world literature. *Neurology* 37:895-904.

Brown, P., M. A. Preece, and R. G. Will. 1992. 'Friendly fire' in medicine: hormones, homografts, and Creutzfeldt- Jakob disease. *Lancet* 340:24-27.
Brown, P., P. Kaur, M. P. Sulima, L. G. Goldfarb, J. Gibbs Cj, and D. C. Gajdusek. 1993. Real and imagined clinicopathological limits of 'prion dementia'. *Lancet* 341:127-129.

Bruce, M. E. and A. G. Dickinson. 1987. Biological evidence that scrapie agent has an independent genome. *Journal of General Virology* 68:79-89.

Bruce, M. E., I. McConnell, H. Fraser, and A. G. Dickinson. 1991. The disease characteristics of different strains of scrapie in sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *Journal of General Virology* 72:595-603.

Bruce, M. E. 1993. Scrapie strain variation and mutation. *British Medical Bulletin* 49:822-838.

Bruce, M. E. and H. Fraser. 1991. Scrapie strain variation and its implications. *Current Topics in Microbiology and Immunology* 172:125-138.

Bueler, H., M. Fischer, Y. Lang, H. Bluethmann, H. P. Lipp, S. J. DeArmond, S. B. Prusiner, M. Aguet, and C. Weissmann. 1992. Normal development and behaviour of mice lacking the neuronal cell- surface PrP protein. *Nature* 356:577-582.

Bueler, H., A. Aguzzi, A. Sailer, R. A. Greiner, P. Autenried, M. Aguet, and C. Weissmann. 1993. Mice devoid of PrP are resistant to scrapie. *Cell* 73:1339-1347.

Butler, D. A., M. R. D. Scott, J. M. Bockman, D. R. Borchelt, A. Taraboulos, K. K. Hsiao, D. T. Kingsbury, and S. B. Prusiner. 1988. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. *Journal of Virology* 62:1558-1564.

Carlson, G. A., D. T. Kingsbury, P. A. Goodman, S. Coleman, S. T. Marshall, S. DeArmond, D. Westaway, and S. B. Prusiner. 1986. Linkage of prion protein and scrapie incubation time genes. *Cell* 46:503-511.

Carlson, G. A., P. A. Goodman, M. Lovett, B. A. Taylor, S. T. Marshall, M. PetersonTorchia, D. Westaway, and S. B. Prusiner. 1988. Genetics and polymorphism of the mouse prion gene complex: control of scrapie incubation time. *Molecular and Cellular Biology* 8:4428-5540.

Carlson, G. A., D. Westaway, S. J. DeArmond, M. PetersonTorchia, and S. B. Prusiner. 1989. Primary structure of prion protein may modify scrapie isolate properties. *Proceedings of the National Academy of Sciences of the United States of America* 86:7475-7479.

Carlson, G. A. 1991. Genetics of prion diversity and host susceptibility. *Current Topics in Microbiology and Immunology* 172:153-164.

Carlson, G. A., K. Hsiao, B. Oesch, D. Westaway, and S. B. Prusiner. 1991. Genetics of prion infections. *Trends in genetics* 7:61-65.

Carlson, G. A., C. Ebeling, S. L. Yang, G. C. Telling, M. Torchia, D. Groth, D. Westaway, S. J. DeArmond, and S. B. Prusiner. 1994. Prion isolate specified allotypic interactions between the cellular and scrapie prion proteins in congenic and transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 91:5690-5694.

Carter, R. L., S. Z. AlSam, R. P. Corbett, and S. Clinton. 1990. A comparative study of immunohistochemical staining for neuron-specific enolase, protein gene product 9.5 and s-100 protein in neuroblastoma, ewing's sarcoma and other round cell tumours in children. *Histopathology* 16:461-467.

Caughey, B., R. E. Race, and B. Chesebro. 1988a. Detection of prion protein mRNA in normal and scrapie-infected tissues and cell lines. *Journal of General Virology* 69:711-716.

Caughey, B., R. E. Race, M. Vogel, M. J. Buchmeier, and B. Chesebro. 1988b. In vitro expression in eukaryotic cells of a prion protein gene cloned from scrapie-infected mouse brain. *Proceedings of the National Academy of Sciences of the United States of America* 85:4657-4661.

Caughey, B., R. E. Race, D. Ernst, M. J. Buchmeier, and B. Chesebro. 1989. Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells. *Journal of Virology* 63:175-181.

Caughey, B., K. Neary, R. Buller, D. Ernst, L. L. Perry, B. Chesebro, and R. E. Race. 1990. Normal and scrapie-associated forms of prion protein differ in their sensitivities to phospholipase and proteases in intact neuroblastoma cells. *Journal of Virology* 64:1093-1101.

Caughey, B., G. J. Raymond, D. Ernst, and R. E. Race. 1991. N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. *Journal of Virology* 65:6597-6603.

Caughey, B. and R. E. Race. 1992. Potent inhibition of scrapie-associated PrP accumulation by congo red. *Journal of Neurochemistry* 59:768-771.

Caughey, B. 1993. Scrapie associated PrP accumulation and its prevention: insights from cell culture. *British Medical Bulletin* 49:860-872.

Caughey, B., D. Ernst, and R. E. Race. 1993. Congo red inhibition of scrapie agent replication. *Journal of Virology* 67:6270-6272.

Caughey, B. and G. J. Raymond. 1993. Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *Journal of Virology* 67:643-650.

Chandler, R. L. 1961. Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet* 1378-1379.

Chesebro, B., R. Race, K. Wehrly, and et. 1985. Identification of scrapie prion protein-specific mRNA in scrapie- infected and uninfected brain. *Nature* 315:331-333.

Chesebro, B., K. Wehrly, B. Caughey, J. Nishio, D. Ernst, and R. Race. 1993. Foreign PrP expression and scrapie infection in tissue culture cell lines. In Transmissible spongiform encephalopathies-impact on animal and human health. F. Brown, editor. Dev. biol. Stand., Basel. 141-140.

Church, G. M. and W. Gilbert. 1984. Genomic sequencing. *Proceedings of the National Academy of Sciences of the United States of America* 81:1991-1995.

Collinge, J., M. A. Whittington, K. C. L. Sidle, C. J. Smith, M. S. Palmer, A. R. Clarke, and J. G. R. Jefferys. 1994. Prion protein is necessary for normal synaptic function. *Nature* 370:295-297.

Collinge, J., M. S. Palmer, K. C. L. Sidle, A. F. Hill, I. Gowland, J. Meads, E. Asante, R. Bradley, L. J. Doey, and P. Lantos. 1995. Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature* 378:779-782.

Dandekar, T., R. Stripecke, N. K. Gray, B. Goossen, A. Constable, H. E. Johansson, and M. W. Hentze. 1991. Identification of a novel iron-responsive element in murine and human erythroid d-aminolevulinic acid synthase mRNA. *EMBO Journal* 10:1903-1909.

Dawson, M., G. A. H. Wells, B. N. J. Parker, and A. C. Scott. 1990. Primary parenteral transmission of bovine spongiform encephalopathy to the pig. *Veterinary record* 126:338.

De Sauvage, F., V. Kruys, O. Marinx, G. Huez, and J. N. Octave. 1992. Alternative polyadenylation of the amyloid protein precursor mRNA regulates translation. *EMBO Journal* 11:3099-3103.

De Silva, R., J. W. Ironside, L. McCardle, T. Esmonde, J. Bell, R. Will, O. Windl, M. Dempster, P. Estibeiro, and R. Lathe. 1994. Neuropathological phenotype and 'prion protein' genotype correlation in sporadic Creutzfeldt-Jakob disease. *Neuroscience Letters* 179:50-52.

DeArmond, S. J., W. C. Mobley, D. L. DeMott, R. A. Barry, J. H. Beckstead, S. B. Prusiner. 1987. Changes in the localization of brain prion proteins during scrapie infection. *Neurology* 37:1271-1280.

DeArmond, S. J., S. L. Yang, A. Lee, R. Bowler, A. Taraboulos, D. Groth, and S. B. Prusiner. 1993. Three scrapie prion isolates exhibit different accumulation patterns of the prion protein scrapie isoform. *Proceedings of the National Academy of Sciences of the United States of America* 90:6449-6453.

Dickinson, A. G., V. M. Meikle, and H. Fraser. 1968. Identification of a gene which controls the incubation period of some strains of scrapie in mice. *Journal of Comparative Pathology* 78:293-299.

Dickinson, A. G. and V. M. Meikle. 1971. Host-genotype and agent effects in scrapie incubation: change in allelic interaction and different strains of agent. *Molecular and General Genetics* 112:73-79.

Dickinson, A. G., H. Fraser, V. M. Meikle, and G. W. Outram. 1972. Competition between different scrapie strains in mice. *Nature New Biology* 237:244-245.

Dickinson, A. G. 1976. Scrapie in sheep and goats. In Slow virus diseaes of animals and man. R. H. Kimberlin, editor. North-Holland, Amsterdam. 209-241.

Dickinson, A. G. and G. W. Outram. 1979. The scrapie replication site hypothesis and its implications for pathogenesis. In Slow transmissible diseases of the nervous system. S. B. Prusiner and W. J. Hadlow, editors. Academic Press, New York. 13-31.

Diedrich, J., S. Wietgrefe, M. Zupancic. 1987. The molecular pathogenesis of astrogliosis in scrapie and Alzheimer's disease. *Microbial Pathogenesis* 2:435-442.

Diedrich, J. F., P. E. Bendheim, Y. S. Kim, R. I. Carp, and A. T. Haase. 1991. Scrapie-associated prion protein accumulates in astrocytes during scrapie infection. *Proceedings of the National Academy of Sciences of the United States of America* 88:375-379.

Diringer, H. 1984. Sustained viremia in experimental hamster scrapie. *Archives of Virology* 82:105-109.

Dlouhy, S. R., K. Hsiao, M. R. Farlow, T. Foroud, P. M. Conneally, P. Johnson, S. B. Prusiner, M. E. Hodes, and B. Ghetti. 1992. Linkage of the Indiana kindred of Gerstmann-Sträussler-Scheinker disease to the prion protein gene. *Nature genetics* 1:64-67.

Dobie, K. W., M. Lee, J. A. Fantesi, E. Graham, A. J. Clark, A. Springbett, R. Lathe, and M. McClenaghan. 1996. Variegated expression in mouse mammary gland is determined by the transgene integration locus. *Proceedings of the National Academy of Sciences of the United States of America.* (In press).

DohUra, K., J. Tateishi, H. Sasaki, T. Kitamoto, and Y. Sasaki. 1989. Protein change at position 102 of prion protein gene is the most common but not the sole mutation related to Gerstmann-Sträussler-Scheinker syndrome. *Biochemical and Biophysical Research Communications* 163:974-979.

Dower, W. J., J. F. Miller, and C. W. Ragsdale. 1988. High efficiency transformation of E. coli by high voltage electroporation. *Nucleic Acids Research* 16:6127.

Duguid, J. R., R. G. Rohwer, and B. Seed. 1988. Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. *Proceedings of the National Academy of Sciences of the United States of America* 85:5738-5742.

Duguid, J. R., C. W. Bohmont, N. Liu, and W. W. Tourtellotte. 1989. Changes in brain gene expression shared by scrapie and Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* 86:7260-7264.

Duguid, J. R. and M. C. Dinauer. 1990. Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucleic Acids Research* 18:2789-2792.

Dutta, S. K., N. Bhattacharyya, R. Parui, and M. Verma. 1990. Expression of DNA sequences containing neuron specific enolase gene in escherichia coli. *Biochemical and Biophysical Research Communications* 173:231-239.

Eklund, C. M., R. C. Kennedy, and W. J. Hadlow. 1967. Pathogenesis of scrapie virus infection in the mouse. *Journal of Infectious Diseases* 117:15.

Endo, T., D. Groth, S. B. Prusiner, and A. Kobata. 1989. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. *Biochemistry (Usa)* 28:8380-8388.

Estibeiro, P., M. Steel, and R. Lathe. 1996. Memory, Alzheimer's disease and the hippocampus: analysis in transgenic mice. *Scandinavian Journal for Laboratory Animal Science* (In Press)

Farquhar, C. F., R. A. Somerville, and L. A. Ritchie. 1989. Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *Journal of Virological Methods* 24:215-221.

Festenstein, R., M. Tolaini, P. Corbella, C. Mamalaki, J. Parrington, M. Fox, A. Miliou, M. Jones, and D. Kioussis. 1996. Locus control region function and heterochromatin-induced position effect variegation. *Science* 271:1123-1125.

Fienberg, A. P. and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* 137:266-267.

Fischer, M., T. Rulicke, A. Raeber, A. Sailer, M. Moser, B. Oesch, S. Brandner, A. Aguzzi and C. Weissmann. 1996. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO journal* 15:1255-1264.

Forloni, G., N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, and F. Tagliavini. 1993. Neurotoxicity of a prion protein fragment. *Nature* 362:543-546.

ForssPetter, S., P. Danielson, and J. G. Sutcliffe. 1986. Neuron-specific enolase: complete structure of rat mRNA, multiple transcriptional start sites, and evidence suggesting post-transcriptional control. *Journal of Neuroscience Research* 16:141-156.

ForssPetter, S., P. E. Danielson, S. Catsicas, E. Battenberg, J. Price, M. Nerenberg, and J. G. Sutcliffe. 1990. Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron* 5:187-197.

Foster, J. D., J. Hope, and H. Fraser. 1993. Transmission of bovine spongiform encephalopathy to sheep and goats. *Veterinary record* 113:339-341.

Fraser, H. and A. G. Dickinson. 1970. Pathogenesis of scrapie in the mouse: the role of the spleen. *Nature.* 226:462-463

Fraser, H. and A. G. Dickinson. 1973. Scrapie in mice: agent-strain differences in the distribution and intensity of grey matter vacuolation. *Journal of Comparative Pathology* 83:29-40.

Fraser, H. and A. G. Dickinson. 1978. Studies of the lymphoreticular system in the pathogenesis of scrapie: the role of spleen and thymus. *Journal of comparative pathology.* 88:563-573

Fraser, H. and A. G. Dickinson. 1985. Targeting of scrapie lesions and spread of the agent via the retino-tectal projection. *Journal of Neuropathology and Experimental Neurology* 346:32-41.

Fraser, H., P. A. McBride, J. R. Scott, and M. E. Bruce. 1986. Infectious degeneration of the nervous system. In Advanced medicine 22. D. R. Triger, editor. Bailliere Tindall, London. 371-384.

Fraser, H. and C. F. Farquhar. 1987. Ionising radiation has no influence on scrapie incubation period in mice. *Veterinary Microbiology* 13:211-223.

Fraser, H., I. McConnell, G. A. H. Wells, and M. Dawson. 1988. Transmission of bovine spongiform encephalopathy to mice. *Veterinary record* 123:472.

Fraser, H. 1993. Diversity in the neuropathology of scrapie-like diseases in animals. *British Medical Bulletin* 49:792-809.

Gabriel, J. M., B. Oesch, H. Kretzschmar, M. Scott, and S. B. Prusiner. 1992. Molecular cloning of a candidate chicken prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 89:9097-9101.

Gajdusek, D. C. and V. Zigas. 1957. Degenerative disease of the central nervous system in New Guinea. The epidemic occurence of 'kuru' in the native population. *New England Journal of Medicine* 257:974-978.

Gajdusek, D. C., C. J. Gibbs, and M. P. Alpers. 1966. Experimental transmission of a kuru-like syndrome to chimpanzees. *Nature* 209:794-796.

Gajdusek, D. C. 1977. Unconventional viruses and the origin and disappearence of kuru. *Science* 197:943-960.

Gajdusek, D. C. 1990. Subacute spongiform encephalopathies: transmissible cerebral amyloidoses caused by unconventional viruses. In Virology. B. Fields,N. and D. Knipe,M., editors. Raven Press,Ltd., New York. 2289-2324.

Gajdusek. 1991. Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the Prnp gene. *Proceedings of the National Academy of Sciences of the United States of America* 88:10926-10930.

Gajdusek, D. C. 1993. Genetic control of nucleation and polymerization of host precursors to infectious amyloids in the transmissible amyloidoses of brain. *British Medical Bulletin* 49:913-931.

Gambetti, P., R. B. Petersen, L. Monari, M. Tabaton, L. Autilio-Gambetti, P. Cortelli, P. Montagna, and E. Lugaresi. 1993. Fatal familial insomnia and the widening spectrum of prion diseases. *British Medical Bulletin* 49:980-994.

Gasset, M., M. A. Baldwin, D. H. Lloyd, J. M. Gabriel, D. M. Holtzman, F. Cohen, R. Fletterick, and S. B. Prusiner. 1992. Predicted alpha-helical regions of the prion protein when synthesized as peptides form amyloid. *Proceedings of the National Academy of Sciences of the United States of America* 89:10940-10944.

Gasset, M., M. A. Baldwin, R. J. Fletterick, and S. B. Prusiner. 1993. Pertrubation of secondary structure of the scrapie prion protein under conditions that alter infectivity. *Proceedings of the National Academy of Sciences of the United States of America* 90:1-5.

Geballe, A. P. and D. R. Morris. 1994. Initiation codons within 5'-leaders of mRNAs as regulators of translation. *Trends in Biochemical Sciences* 19:159-164.

Gibbs, C. J., A. Joy, R. Heffner, and et. 1985. Clinical and pathological features and laboratory confirmation of Creutzfeldt-Jakob disease in a recipient of pituitary-derived human growth hormone. *New England Journal of Medicine* 313:734-738.

Gizang-Ginsberg, E. and E. B. Ziff. 1990. Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-fos. *Genes and development* 4:477-491.

Goldfarb, L. G., E. Mitrova, P. Brown, B. H. Toh, and D. C. Gajdusek. 1990. Mutation of codon 200 of scrapie amyloid protein gene in two clusters of Creutzfeldt-Jakob disease in Slovakia. *Lancet* 336:514-515.

Goldfarb, L. G., P. Brown, W. R. McCombie, D. Goldgaber, G. D. Swergold, P. R. Wills, L. Cervenakova, H. Baron, J. Gibbs Cj, and D. C.

Goldfarb, L. G., P. Brown, E. Mitrova, L. Cervenokova, L. Goldin, A. D. Korczyn, J. Chapman, S. Galvez, L. Cartier, R. Rubenstein, and D. C. Gajdusek. 1991b. Creutzfeldt-Jakob disease associated with the Prnp codon 200(lys) mutation: an analysis of 45 families. *European Journal of Epidemiology* 7:477-486.

Goldfarb, L. G., M. Haltia, P. Brown, A. Nieto, J. Kovanen, W. R. McCombie, S. Trapp, and D. Carleton Gajdusek. 1991c. New mutation in scrapie amyloid precursor gene (at codon 178) in Finnish Creutzfeldt-Jakob kindred. *Lancet* 337:425.

Goldfarb, L. G., R. B. Petersen, M. Tabaton, P. Brown, A. C. LeBlanc, P. Montagna, P. Cortelli, J. Julien, C. Vital, W. W. Pendelbury, M. Haltia, P. R. Wills, J. J. Hauw, P. E. McKeever, L. Monari, B. Schrank, G. D. Swergold, L. AutilioGambetti, D. Carleton Gajdusek, and et al. 1992. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science* 258:806-808.

Goldfarb, L. G., P. Brown, M. Haltia, J. Ghiso, B. Frangione, and D. C. Gajdusek. 1993a. Synthetic peptides corresponding to different mutated regions of the amyloid gene in familial Creutzfeldt-Jakob disease show enhanced in vitro formation of morphologically different amyloid fibrils. *Proceedings of the National Academy of Sciences of the United States of America* 90:4451-4454.

Goldfarb, L. G., P. Brown, B. Little, L. Cervenakova, K. Kenney, C. J. Gibbs, and D. C. Gajdusek. 1993b. A new (two-repeat) octapeptide coding insert mutation in Creutzfeldt-Jakob disease. *Neurology* 43:2392-2394.

Goldmann, W., N. Hunter, J. D. Foster, J. M. Salbaum, K. Beyreuther, and J. Hope. 1990. Two alleles of a neural protein gene linked to scrapie in sheep. *Proceedings of the National Academy of Sciences of the United States of America* 87:2476-2480.

Goldmann, W., N. Hunter, G. Benson, J. D. Foster, and J. Hope. 1991a. Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the sip gene. *Journal of General Virology* 72:2411-2417.

Goldmann, W., N. Hunter, T. Martin, M. Dawson, and J. Hope. 1991b. Different forms of the bovine PrP gene have five or six copies of a short, GC-rich element within the protein-coding exon. *Journal of General Virology* 72:201-204.

Goldmann, W. 1993. PrP gene and its association with spongiform encephalopathies. *British Medical Bulletin* 49:839-859.

Goldmann, W., N. Hunter, G. Smith, J. Foster, and J. Hope. 1994. PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *Journal of General Virology* 75:989-995.

Grima, B., A. Lamouroux, C. Boni, and et al. 1987. A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. *Nature* 326:707-711.

Hadlow, W. J. 1959. Scrapie and Kuru. *Lancet* 2:289-290.

Hadlow, W. J., R. C. Kennedy, and R. E. Race. 1982. Natural infection of Suffolk sheep with scrapie virus. *Journal of Infectious Diseases* 146:657-664.

Hadlow, W. J., R. E. Race, and R. C. Kennedy. 1987. Temporal distribution of transmissible mink encephalopathy virus in mink inoculated subcutaneously. *Journal of Virology* 61:3235-3240.

Haile,D. J., T. A. Roualt, C. K. Tang, J. Chin, J. B. Harford, and R. D. Klausner. 1992. Reciprocal control of RNA-binding and aconitase activity in the regulation of the iron-responsive element binding protein: role of the iron-sulfur cluster. *Proceedings of the National Academy of Sciences of the United States of America* 89:7536-7540.

Hanson, R. P., R. J. Eckroade, R. F. Marsh, G. M. Zurhein, C. L. Kanitz, and D. P. Gustafson. 1971. Susceptibility of mink to sheep scrapie. *Science* 172:859-861.

Harris, D. A., D. L. Falls, F. A. Johnson, and G. D. Fischbach. 1991. A prion-like protein from chicken brain copurifies with an acetylcholine receptor-inducing activity. *Proceedings of the National Academy of Sciences of the United States of America* 88:7664-7668.

Hatsough, G. R. and D. Burger. 1965. Encephalopathy of mink. I. Epizootiologic and clinical observations. *Journal of Infectious Diseases* 115:387-392.

Hay, B., R. A. Barry, I. Lieberburg, S. B. Prusiner, and V. R. Lingappa. 1987a. Biogenesis and transmembrane orientation of the cellular isoform of the scrapie prion protein. *Molecular and Cellular Biology* 7:914-920.
Hay, B., S. B. Prusiner, and V. R. Lingappa. 1987b. Evidence for a secretory form of the cellular prion protein. *Biochemistry (Usa)* 26:8110-8115.

Hecker, R., A. Taraboulos, M. Scott, K. M. Pan, S. L. Yang, M. Torchia, K. Jendroska, S. J. DeArmond, and S. B. Prusiner. 1992. Replication of distinct scrapie prion isolates is region specific in brains of transgenic mice and hamsters. *Genes and development* 6:1213-1228.

Hitoshi, S., H. Nagura, H. Yamanouchi, and T. Kitamoto. 1993. Double mutations at codon 180 and codon 232 of the Prnp gene in an apparently

sporadic case of Creutzfeldt-Jakob disease. *Journal of the Neurological Sciences* 120:208-212.

Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the mouse embryo. A laboratory manual. Cold Spring Harbor Laboratory, New York.

Hope, J., L. J. D. Morton, C. F. Fraquar, G. Multhaup, K. Beyreuther, and R. H. Kimberlin. 1986. The major polypeptide of scrapie associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *EMBO Journal* 5:2591-2597.

Hope, J. and N. Hunter. 1988. Scrapie-associated fibrils, PrP protein and the Sinc gene. *Ciba Foundation Symposium* 135:146-163.

Hope, J., L. J. D. Reekie, N. Hunter, G. Multhaup, K. Beyreuther, H. White, A. C. Scott, M. J. Stack, M. Dawson, and G. A. H. Wells. 1988. Fibrils from brains of cows with new cattle disease contain scrapie-associated protein. *Nature* 336:390-392.

Hope,J., G. Multhaup, L. J. Reekie, R. H. Kimberlin and K. Beyreuther. 1988. Molecular pathology of scrapie-associated fibril protein (PrP) in mouse brain affected by the ME7 strain of scrapie. *European journal of Biochemistry* 172:271-277

Hope, J. and H. Baybutt. 1991. The key role of the nerve membrane protein PrP in scrapie-like diseases. *Seminars in The Neurosciences* 3:165-171.

Hope, J. 1993. The biochemistry, protein chemistry and molecular biology of BSE. *Journal of Am Medical Association*

Horiuchi, M., N. Yamazaki, T. Ikeda, N. Ishiguro, and M. Shinagawa. 1995. A cellular form of prion protein (PrP^C) exists in many non-neuronal tissues of sheep. *Journal of General Virology* 76:2583-2587.

Hsiao, K., H. F. Baker, T. J. Crow, M. Poulter, F. Owen, J. D. Terwilliger, D. Westaway, J. Ott, and S. B. Prusiner. 1989. Linkage of a prion protein missense variant to Gerstmann-Sträussler syndrome. *Nature* 338:342-345.

Hsiao, K., M. Scott, D. Foster, S. J. DeArmond, D. Groth, H. Serban, and S. B. Prusiner. 1991. Spontaneous neurodegeneration in transgenic mice with prion protein codon 101 proline>leucine substitution. *Annals of the New York Academy of Sciences* 640:166-170.

Hsiao, K., S. R. Dlouhy, M. R. Farlow, C. Cass, M. Da Costa, P. M. Conneally, M. E. Hodes, B. Ghetti, and S. B. Prusiner. 1992. Mutant prion proteins in Gerstmann-Straussler-Scheinker disease with neurofibrillary tangles. *Nature genetics* 1:68-71.

Hsiao, K. K., D. Groth, M. Scott, S. L. Yang, H. Serban, D. Rapp, D. Foster, M. Torchia, S. J. DeArmond, and S. B. Prusiner. 1994. Serial transmission in rodents of neurodegeneration from transgenic mice expressing mutant prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 91:9126-9130.

Huang, Z., J. M. Gabriel, M. A. Baldwin, R. J. Fletterick, S. B. Prusiner, and F. E. Cohen. 1994. Proposed three-dimensional structure for the cellular prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 91:7139-7143.

Hung, A. Y., E. H. Koo, C. Haass, and D. J. Selkoe. 1992. Increased expression of β-amyloid precursor protein during neuronal differentiation is not accompanied by secretory cleavage. *Proceedings of the National Academy of Sciences of the United States of America* 89:9439-9443.

Hunter, N., J. D. Foster, A. G. Dickinson, and J. Hope. 1989. Linkage of the gene for the scrapie-associated fibril protein (PrP) to the Sip gene in Cheviot sheep. *Veterinary record* 124:364-366.

Hunter, N., J. C. Dann, A. D. Bennett, R. A. Somerville, I. McConnell, and J. Hope. 1992. Are sinc and the PrP gene congruent? evidence from PrP gene analysis in sinc congenic mice. *Journal of General Virology* 73:2751-2755.

Hunter, N., J. C. Manson, F. C. Charleson, and J. Hope. 1994. Comparison of expression patterns of PrP mRNA in the developing sheep and mouse. *Annals of the New York Academy of Sciences* 724:353-354.

Iwasaki, M., K. Okumura, Y. Kondo, T. Tanaka, and H. Igarashi. 1992. cDNA cloning of a novel heterogeneous nuclear ribonucleoprotein gene homologue in caenorhabditis elegans using hamster prion protein cDNA as a hybridization probe. *Nucleic Acids Research* 20:4001-4007.

Jackson, R. J. 1993. Cytoplasmic regulation of mRNA function: the importance of the untranslated region. *Cell* 74:9-14.

Jeffrey, M., C. M. Goodsir, M. Bruce, P. A. McBride, J. R. Scott and W. G. Halliday. 1994a. Correlative light and electron microscopy studies of PrP localisation in 87V scrapie. *Brain research* 656:329-343.

Jeffrey, M., C. M. Goodsir, M. Bruce, P. A. McBride, N. Fowler and J.R. Scott. 1994b. Murine scrapie-infected neurons in vivo release excess prion protein into the extracellular space. *Neuroscience Letters* 174:39-42.

Jeffrey, M., C. M. Goodsir, M. Bruce, P. A. McBride and C. Farquhar. 1994c. Morphogenesis of amyloid plaques in 87V murine scrapie. *Neuropathology and Applied Neurobiology.* 20:535-542

Kandel, E. R., J. H. Schwartz, and T. M. Jessel. 1991. Principles of neural science. Elsevier Science Publishing, Amsterdam. 215 pp.

Kaneda, N., T. Sasaoka, K. Kobayashi, K. Kiuchi, I. Nagatsu, Y. Kurosawa, K. Fujita, M. Yokoyama, T. Nomura, M. Katsuki, and T. Nagatsu. 1991. Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* 6:583-594.

Kascsak, R. J., R. Rubenstein, P. A. Merz, M. Tonna-DeMasi, R. Fersko, R. I. Carp, H. M. Wisniewski, and H. Diringer. 1987. Mouse polyclonal and monoclonal antibody to scrapie associated fibril proteins. *Journal of Virology* 61:3688-3693.

Kascsak, R. J., R. Rubenstein, and R. I. Carp. 1991. Evidence for biological and structural diversity among scrapie strains. *Current Topics in Microbiology and Immunology* 172:139-152.

Kellings, K., N. Meyer, C. Mirenda, S. B. Prusiner, and D. Riesner. 1992. Further analysis of nucleic acids in purified scrapie prion preparation by improved return refocusing gel electrophoresis. *Journal of General Virology* 73:1025-1029.

Kim, Y. S., R. I. Carp, and S. M. Callahan. 1987. Incubation periods and survival times for mice injected stereotaxically with three scrapie strains in different brain regions. *Journal of General Virology* 68:695-702.

Kim, Y. S., R. I. Carp, S. Callahan, and H. M. Wisniewski. 1990a. Incubation periods and histopathological changes in mice injected stereotaxically in different brain areas with the 87V scrapie strain. *Acta Neuropathologica* 80:388-392.

Kim, Y. S., R. I. Carp, S. M. Callahan, and H. M. Wisniewski. 1990b. Pathogenesis and pathology of scrapie after stereotactic injection of strain 221 in intact and bisected cerebella. *Journal of Neuropathology and Experimental Neurology* 49:114-121.

Kimberlin, R. H. and C. A. Walker. 1978. Pathogenesis of mouse scrapie: effect of route of inoculation on infectivity titres and dose-response curves. *Journal of Comparative Pathology* 88:39-47.

Kimberlin, R. H. and C. A. Walker. 1979a. Pathogenesis of scrapie: agent multiplication in the brain at first and second passage of hamster scrapie in mice. *Journal of General Virology* 42:107-117.

Kimberlin, R. H. and C. A. Walker. 1979b. Pathogenesis of mouse scrapie: dynamics of agent replication in spleen, spinal cord and brain after infection by different routes. *Journal of Comparative Pathology* 89:551-562.

Kimberlin, R. H. and C. A. Walker. 1983. Invasion of the CNS by scrapie agent and its spread to different parts of the brain. In Virus non-conventionalles et affections du systeme nerveux central. F. Cathala, editor. Masson, Paris. 17-33.

Kimberlin, R. H., S. Cole, and C. A. Walker. 1987a. Pathogenesis of scrapie is faster when infection is intraspinal instead of intracerebral. *Microbial Pathogenesis* 2:405-415.

Kimberlin, R. H., S. Cole, and C. A. Walker. 1987b. Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. *Journal of General Virology* 68:1875-1881.

Kimberlin, R. H. and C. A. Walker. 1988a. Pathogenesis of experimental scrapie. In Novel infectious agents and the central nervous system. Wiley, editor. Ciba Foundation Symposium, Chichester. 37-62.

Kimberlin, R. H. and C. A. Walker. 1988b. Transport, targeting and replication of scrapie in the CNS. In Unconventional viruses and central nervous system dseases . L. A. Court and et al, editors. Atelier d'Art Graphique, Abbaye de Melleray. 547-562.

Kimberlin, R. H. and C. A. Walker. 1989a. Pathogenesis of scrapie in mice after intragastric infection. *Virus Research* 12:213-220.

Kimberlin, R. H. and C. A. Walker. 1989b. The role of the spleen in the neuroinvasion of scrapie in mice. *Virus Research* 12:201-212.

Kimberlin, R. H., C. A. Walker, and H. Fraser. 1989. The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. *Journal of General Virology* 70:2017-2025.

Kimberlin, R. H. 1990a. Transmissible encephalopathies in animals. *Canadian Journal of Veterinary Research* 54:30-37.

Kimberlin, R. H. 1990b. Unconventional 'slow' viruses. In Topley & . Wilson's principles of bacteriology, virology and immunity. Volume 4. Virology. L. H. Collier and M. C. Timbury, editors. Edward Arnold, London Melbourne Auckland. 672-693.

Kimberlin, R. H. and C. A. Walker. 1977. Characteristics of a short incubation model of scrapie in golden hamster. *Journal of General Virology* 34:295-304.

Kirkwood, J. M., A. A. Cunningham, G. A. Wells, J. W. Wilesmith and J. E. Barnett. 1993. Spongiform encephalopathy in a herd of greater kudu (Tragelaphus strepsiceros): epidemiological observations. *Veterinary Record* 133: 360-364.

Kitamoto, T., T. Muramoto, S. Mohri, K. DohUra, and J. Tateishi. 1991. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. *Journal of Virology* 65:6292-6295.

Kitamoto, T., R. Iizuka, and J. Tateishi. 1993a. An amber mutation of the prion protein in Gerstmann-Sträussler syndrome with mutant PrP plaques. *Biochemical and Biophysical Research Communications* 192:525-531.

Kitamoto, T., M. Ohta, K. DohUra, S. Hitoshi, Y. Terao, and J. Tateishi. 1993b. Novel missense variants of prion protein in Creutzfeldt-Jakob disease or Gerstmann-Sträussler syndrome. *Biochemical and Biophysical Research Communications* 191:709-714.

Klatzo, I., D. C. Gajdusek, and V. Zigas. 1959. Pathology of kuru. *Laboratory Investigation*. 8:799-847.

Kocisko, D. A., J. H. Come, S. A. Priola, B. Chesebro, G. J. Raymond, P. T. Lansbury, and B. Caughey. 1994. Cell-free formation of protease-resistant prion protein. *Nature* 370:471-474.

Kocisko, D. A., S. A. Priola, G. J. Raymond, B. Chesebro, J. Lansbury Pt, and B. Caughey. 1995. Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proceedings of the National Academy of Sciences of the United States of America* 92:3923-3927.

Koeller, D. M., J. L. Casey, M. W. Hentze, E. M. Gerhardt, L. L. Chan, R. D. Klausner, and J. B. Harford. 1989. A cytosolic protein binds to structural elements within the iron regulatory region of transferrin receptor mRNA. *Proceedings of the National Academy of Sciences of the United States of America* 86:3574-3578.

Kozak, M. 1987a. At least six nucleotides preceding the AUG codon enhance translation in mammalian cells. *Journal of Molecular Biology* 196:947-950.

Kozak, M. 1987b. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. *Molecular and Cellular Biology* 3438-3445.

Kozak, M. 1989. The scanning model for translation: an update. *J. Cell. Biol.* 108:229-241.

Kozak, M. 1991a. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *Journal of Biological Chemistry* 266:19867-19870.

Kozak, M. 1991b. An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell. Biol.* 115:887-903.

Kretzschmar, H. A., S. B. Prusiner, L. E. Stowring, and S. J. DeArmond. 1986a. Scrapie prion proteins are synthesized in neurons. *American Journal of Pathology* 122:1-5.

Kretzschmar, H. A., L. E. Stowring, D. Westaway, W. H. Stubblebine, S. B. Prusiner, and S. J. DeArmond. 1986b. Molecular cloning of a human prion protein cDNA. *DNA* 5:315-324.

Kretzschmar, H. A., G. Honold, F. Seitelberger, M. Feucht, P. Wessely, P. Mehraein, and H. Budka. 1991. Prion protein mutation in family first reported by Gerstmann, Sträussler, and Scheinker (7). *Lancet* 337:1160.

Kretzschmar, H. A., M. Neumann, G. Riethmuller, and S. B. Prusiner. 1992. Molecular cloning of a mink prion protein gene. *Journal of General Virology* 73:2757-2761.

Kretzschmar, H. A. 1993. Human prion diseases (spongiform encephalopathies). *Archives of Virology Supplement* 7:261-293.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature* 227:680-685.

Lantos, P. L. 1992. From slow virus to prion: a review of transmissible spongiform encephalopathies. *Histopathology* 20:1-11.

Laplanche, J. L., J. Chatelain, M. Dussaucy, C. Bounneau, J. M. Launay, J. P. Brandel, N. DelasnerieLaupretre, and H. C. Grant. 1993. Inherited prion disease (14). *British Medical Journal* 306:794-795.

Lathe, R. and J. J. Mullins. 1993. Transgenic animals as models for human disease - report of an EC study group. *Transgenic research* 2:286-299.

Lathe, R. and R. G. M. Morris. 1994. Analysing brain function and dysfunction in transgenic animals. *Neuropathology and Applied Neurobiology* 20:350-358.

Lazarini, F., J. P. Deslys, and D. Dormont. 1991. Regulation of the glial fibrillary acidic protein, β actin and prion protein mRNAs during brain development in mouse. *Molecular Brain Research* 10:343-346.

Le Bourdelles, B., S. Boularand, C. Boni, P. Horellou, S. Dumas, B. Grima, and J. Mallet. 1988. Analysis of the 5' region of the human tyrosine hydroxylase gene: combinatorial patterns of exon splicing generate multiple regulated tyrosine hydroxylase isoforms. *Journal of Neurochemistry* 50:988-991.

Lewis, E. J. and D. M. Chikaraishi. 1987. Regulated expression of tyrosine hydroxylase gene by epidermal growth factor. *Mol. Cell. Biol.* 7:3332-3336.

Lewis, E. J., C. A. Harrington, and D. M. Chikaraishi. 1987. Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. *Proceedings of the National Academy of Sciences of the United States of America* 84:3550-3554.

Liao, Y. C., R. V. Lebo, G. A. Clawson, and E. A. Smuckler. 1986. Human prion protein cDNA: molecular cloning, chromosomal mapping, and biological implications. *Science* 233:364-367.

Lledo, P., P. Tremblay, S. J. DeArmond, S. B. Prusiner, and R. A. Nicoll. 1996. Mice deficient for prion protein exhibit normal neuronal excitability and synaptic transmission in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America* (In Press)

Locht, C., B. Chesebro, R. Race, and J. M. Keith. 1986. Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with scrapie agent. *Biochemistry* 83:6372-6376.

Lopez, C. D., C. S. Yost, S. B. Prusiner, R. M. Myers, and V. R. Lingappa. 1990. Unusual topogenic sequence directs prion protein biogenesis. *Science* 248:226-229.

Lowenstein, D. H., D. A. Butler, D. Westaway, M. P. McKinley, S. J. DeArmond, and S. B. Prusiner. 1990. Three hamster species with

different scrapie incubation times and neuropathological features encode distinct prion proteins. *Molecular and Cellular Biology* 10:1153-1163.

Manson, J., P. McBride, and J. Hope. 1992a. Expression of the PrP gene in the brain of Sinc congenic mice and its relationship to the development of scrapie. *Neurodegeneration* 1:45-52.

Manson, J., J. D. West, V. Thomson, P. McBride, M. H. Kaufman, and J. Hope. 1992b. The prion protein gene: a role in mouse embryogenesis? *Development* 115:117-122.

Manson, J. C., A. R. Clarke, M. L. Hooper, L. Aitchison, I. McConnell, and J. Hope. 1994. 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. *Molecular Neurobiology* 8:121-127.

Manson, J. C., J. Hope, A. R. Clarke, A. Johston, C. Black, and N. MacLeod. 1995. PrP gene dosage and long term potentiation. *Neurodegeneration* 4:113-115.

Manuelidis, L., T. Sklaviadis, and E. E. Manuelidis. 1987. Evidence suggesting that PrP is not the infectious agent in Creutzfeldt-Jakob disease. *EMBO Journal* 6:341-347.

Marangos, P. J., D. E. Schmechel, A. M. Parma, and F. K. Goodwin. 1980. Developmental profile of neuron-specific (NSE) and non-neuronal enolase (NNE) . *Brain Research* 190:185-193.

Marsh, R. F. and R. H. Kimberlin. 1975. Comparison of scrapie and transmissible mink encephalopathy in hamsters. II. Clinical sighs, pathology, and pathogenesis. *Journal of Infectious Diseases* 131:104-110.

Masters, C. L., J. O. Harris, D. C. Gajdusek, C. J. Gibbs, C. Bernoulli, and D. M. Asher. 1979. Creutzfeldt-Jakob disease: pattern of worldwide occurence and the significance of familial and sporadic clustering. *Annals of Neurology* 5:177-188.

Masters, C. L., D. C. Gajdusek, and C. J. Gibbs. 1981a. Creutzfeldt-Jakob disease virus isolations from the Gerstmann-Sträussler syndrome with analysis of the various forms of amyloid plaque deposition in the virus-induced spongiform encephalopathies. *Brain* 104:559-588.

Masters, C. L., D. C. Gajdusek, and C. J. Gibbs. 1981b. The familial occurence of Creutzfeldt-Jakob disease and Alzheimer's disease. *Brain* 104:535-558.

McKinley, M. P., D. C. Bolton, and S. B. Prusiner. 1983. A protease-resistant protein is a structural component of the scrapie prion. *Cell* 35:57-62.

McKinley, M. P., B. Hay, V. R. Lingappa, and et. 1987. Developmental expression of prion protein gene in brain. *Developmental Biology* 121:105-110.

McKinley, M. P., F. M. Longo, J. S. Valletta, F. Rahbar, R. L. Neve, S. B. Prusiner, and W. C. Mobley. 1990. Nerve growth factor induces gene expression of the prion protein and beta-amyloid protein precursor in the developing hamster central nervous system. *Progress in Brain Research* 86:227-238.

Medori, R., H. J. Tritschler, A. LeBlanc, F. Villare, V. Manetto, C. Hsiao Ying, R. Xue, S. Leal, P. Montagna, P. Cortelli, P. Tinuper, P. Avoni, M. Mochi, A. Baruzzi, J. J. Hauw, J. Ott, E. Lugaresi, L. AutilioGambetti, and P. Gambetti. 1992. Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. *New England Journal of Medicine* 326:444-449.

Merrick, W. 1992. Mechanism and regulation of eukaryotic protein synthesis. *Microbiological Reviews* 291-315.

Merz, P. A., R. A. Somerville, H. M. Wisniewski, and K. Iqbal. 1981. Abnormal fibrils from scrapie-infected brain. *Acta Neuropathologica* 54:63-74.

Meyer, N., V. Rosenbaum, B. Schmidt, K. Gilles, C. Mirenda, D. Groth, S. B. Prusiner, and D. Riesner. 1991. Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids. *Journal of General Virology* 72:37-49.

Meyer, R. K., M. P. McKinley, K. A. Bowman, M. B. Braunfeld, R. A. Barry, and S. B. Prusiner. 1986. Separation and properties of cellular and scrapie prion proteins. *Proceedings of the National Academy of Sciences of the United States of America* 83:2310-2314.

Millson, G. C., R. H. Kimberlin, E. J. Manning, and S. C. Collis. 1979. Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. *Veterinary Microbiology* 4:89-99.

Min, N., T. H. Joh, K. S. Kim, C. Peng, and J. H. Son. 1994. 5' upstream DNA sequence of the rat tyrosine hydroxylase gene directs high-level and tissue-specific expression to catecholaminergic neurons in the

central nervous system of transgenic mice. *Molecular Brain Research* 27:281-289.

Miyazono, M., T. Kitamoto, K. DohUra, T. Iwaki, and J. Tateishi. 1992. Creutzfeldt-Jakob disease with codon 129 polymorphism (valine): a comparative study of patients with codon 102 point mutation or without mutations. *Acta Neuropathologica* 84:349-354.

Mobley, W. C., R. L. Neve, S. B. Prusiner, and M. P. McKinley. 1988. Nerve growth factor increases mRNA levels for the prion protein and the beta-amyloid protein precursor in developing hamster brain. *Proceedings of the National Academy of Sciences of the United States of America* 85:9811-9815.

Mould, D. L. and W. Smith. 1995. The causal agent of scrapie. *Journal of Comparative Pathology* 72:97-105.

Murdoch, G. H., T. Sklaviadis, E. E. Manuelidis, and L. Manuelidis. 1990. Potential retroviral RNAs in Creutzfeldt-Jakob disease. *Journal of Virology* 64:1477-1486.

Narang, H. K., D. M. Asher, and D. C. Gajdusek. 1988. Evidence that DNA is present in abnormal tubulofilamentous structures found in scrapie. *Proceedings of the National Academy of Sciences of the United States of America* 85:3575-3579.

Narang, H. K. 1990. Detection of single-stranded DNA in scrapie-infected brain by electron microscopy. *Journal of Molecular Biology* 216:469-473.

Oesch, B., D. Westaway, M. Walchli, and *et al.* 1985. A cellular gene encodes scrapie PrP 27-30 protein. *Cell* 40:735-746.

Old, R. W. and S. B. Primrose. 1985. Cutting and joining DNA molecules. In Principles of gene manipulation. Blackwell Scientific Publications, Oxford, London, Edinburgh, Boston, Palo Alto, Melbourne. 20-44.

Ostareck-Lederer, A., D. H. Ostareck, N. Standart, and B. J. Thiele. 1994. Translation of 15-lipoxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3' untranslated region. *EMBO Journal* 13:1476-1481.

Owen, F., M. Poulter, T. Shah, J. Collinge, R. Lofthouse, H. Baker, R. M. Ridley, J. McVey, and T. J. Crow. 1990. An in-frame insertion in the prion protein gene in familial Creutzfeldt-Jakob disease. *Molecular Brain Research* 7:273-276.

Owen, F., M. Poulter, J. Collinge, M. Leach, T. Shah, R. Lofthouse, Y. Chen, T. J. Crow, A. E. Harding, J. Hardy, and M. N. Rossor. 1991. Insertions in the prion protein gene in atypical dementias. *Experimental Neurology* 112:240-242.

Ozel, M. and H. Diringer. 1994. Small virus-like structure in fractions from scrapie hamster brain. *Lancet* 343:894-895.

Palmer, M. S., A. J. Dryden, J. T. Hughes, and J. Collinge. 1991. Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease. *Nature* 352:340-342.

Palmer, M. S., S. P. Mahal, T. A. Campbell, A. F. Hill, K. C. L. Sidle, J. L. Laplanche, and J. Collinge. 1993. Deletions in the prion protein gene are not associated with CJD. *Human Molecular Genetics* 2:541-544.

Palmiter, R. D. and R. L. Brinster. 1986. Germ-line transformation of mice. *Annual Review of Genetics* 20:465-499.

Pan, K. M., M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R. J. Fletterick, F. E. Cohen, and S. B. Prusiner. 1993. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proceedings of the National Academy of Sciences of the United States of America* 90:10962-10966.

Pattison, I. H. and G. C. Millson. 1960. Further observations on the experimental production of scrapie in goats and sheep. *Journal of Comparative Pathology* 182:182-193.

Pattison, I. H. 1965. The relative susceptibility of sheep, goats and mice to two types of the scrapie agent. *Research in Veterinary Science* 7:207-212.

Pattison, I. H. and G. C. Millson. 1961. Scrapie produced experimentally in goats with special reference to the clinical syndrome. *Journal of Comparative Pathology* 71:101-108.

Pattison, I. H. and G. C. Millson. 1962. Distribution of the scrapie agent in the tissues of experimentally inoculated goats. *Journal of Comparative Pathology* 72:233-244.

Pocchiari, M., M. Salvatore, F. Cutruzzola, M. Genuardi, C. T. Allocatelli, C. Masullo, G. Macchi, G. Alema, S. Galgani, X. You Geng, R. Petraroli, M. C. Silvestrini, and M. Brunori. 1993. A new point mutation of the prion protein gene in Creutzfeldt-Jakob disease. *Annals of Neurology* 34:802-807.

Priola, S. A. and B. Caughey. 1994. Inhibition of scrapie associated PrP accumulation. Probing for the role of glycosaminoglycans in amyloidogenesis. *Molecular Neurobiology* 8:113-120.

Priola, S. A., B. Caughey, R. E. Race, and B. Chesebro. 1994a. Heterologous PrP molecules interfere with accumulation of protease-resistant PrP in scrapie-infected murine neuroblastoma cells. *Journal of Virology* 68:4873-4878.

Priola, S. A., B. Caughey, G. J. Raymond, and B. Chesebro. 1994b. Prion protein and the scrapie agent: in vitro studies in infected neuroblastoma cells. *Infectious Agents and Disease* 3:54-58.

Priola, S. A., B. Caughey, K. Wehrly, and B. Chesebro. 1995. A 60-kDa prion protein (PrP) with properties of both the normal and scrapie-associated forms of PrP. *Journal of Biological Chemistry* 270:3299-3305.

Prusiner, S. B., W. J. Hadlow, D. E. Garfin, S. P. Cochran, J. R. Baringer, R. E. Race, and C. M. Eklund. 1978. Partial purification and evidence for multiple molecular forms of the scrapie agent. *Biochemistry* 17:4993-4999.

Prusiner, S. B., D. F. Groth, C. Bildstein, and et al. 1980a. Electrophoretic properties of the scrapie agent in agarose gels. *Proceedings of the National Academy of Sciences of the United States of America* 77:2984-2988.

Prusiner, S. B., D. F. Groth, S. P. Cochran, F. R. Masiarz, M. P. McKinley, and H. M. Martinez. 1980b. Molecular properties, partial purification and assay by incubation period measurements of the hamster scrapie agent. *Biochemistry* 19:4883-4891.

Prusiner, S. B., M. P. McKinley, D. F. Groth, and et. 1981. Scrapie agent contains a hydrophobic protein. *Proceedings of the National Academy of Sciences of the United States of America* 78:6675-6679.

Prusiner, S. B. 1982. Novel proteinaceous infectious particles cause scrapie. *Science* 216:136-144.

Prusiner, S. B., D. C. Bolton, D. F. Groth, and et al. 1982. Further purification and characterization of scrapie prions. *Biochemistry (USA)* 21:6942-6950.

Prusiner, S. B., D. F. Groth, D. C. Bolton, and et. 1984. Purification and structural studies of a major scrapie prion protein. *Cell* 38:127-134.

Prusiner, S. B., M. Scott, D. Foster, K. M. Pan, D. Groth, C. Mirenda, M. Torchia, S. L. Yang, D. Serban, G. A. Carlson, P. C. Hoppe, D. Westaway, and S. J. DeArmond. 1990. Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* 63:673-686.

Prusiner, S. B. 1991. Molecular biology and transgenetics of prion diseases. *Critical Reviews in Biochemistry and Molecular Biology* 26:397-438.

Prusiner, S. B. 1993a. Transgenetics and cell biology of prion diseases: investigations of PrP^{Sc} synthesis and diversity. *British Medical Bulletin* 49:873-912.

Prusiner, S. B. 1993b. Genetic and infectious prion diseases. *Archives of Neurology* 50:1129-1153.

Prusiner, S. B., D. Groth, A. Serban, R. Koehler, D. Foster, M. Torchia, D. Burton, S. L. Yang, and S. J. DeArmond. 1993. Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proceedings of the National Academy of Sciences of the United States of America* 90:10608-10612.

Prusiner, S. B. 1994a. Neurodegeneration in humans caused by prions. *Western Journal of Medicine* 161:264-272.

Prusiner, S. B. 1994b. Inherited prion diseases. *Proceedings of the National Academy of Sciences of the United States of America* 91:4611-4614.

Prusiner, S. B. and S. J. DeArmond. 1994. Prion diseases and neurodegeneration. *Annual Review of Neuroscience* 17:311-339.

Prusiner, S. B. and K. K. Hsiao. 1994. Human prion diseases. *Annals of Neurology* 35:385-395.

Puckett, C., P. Concannon, C. Casey, and L. Hood. 1991. Genomic structure of the human prion protein gene. *American Journal of Human Genetics* 49:320-329.

Quon, D., Y. Wang, R. Catalano, J. M. Scardina, K. Mukarami, and B. Cordell. 1991. Formation of β-amyloid protein deposits in brains of transgenic mice. *Nature* 352:239-241.

Race, R. E., L. H. Fadness, and B. Chesebro. 1987. Characterization of scrapie infection in mouse neuroblastoma cells. *Journal of General Virology* 68:1391-1399.

Race, R. E., S. A. Priola, R. Bessen, D. Ernst, J. Dockter, G. F. Rall, L. Mucke, B. Chesebro, and M. B. A. Oldstone. 1995. Neuron-specific expression of a hamster prion protein minigene in transgenic mice induces susceptibility to hamster scrapie agent. *Neuron* 15:1183-1191.

Raeber, A. J., D. R. Borchelt, M. Scott, and S. B. Prusiner. 1992. Attempts to convert the cellular prion protein into the scrapie isoform in cell-free systems. *Journal of Virology* 66:6155-6163.

Rastinejad, F. and H. M. Blau. 1993. Genetic complementation reveals a novel regulatory role of 3' untranslated regions in growth and differentiation. *Cell* 72:903-917.

Rastinejad, F., M. J. Conboy, T. A. Rando, and H. M. Blau. 1993. Tumor suppression by RNA from the 3' untranslated region of α-tropomyosin. *Cell* 75:1107-1117.

Ripoll, L., J. L. Laplanche, M. Salzmann, A. Jouvet, B. Planques, M. Dussausy, J. Chatelain, P. Beaudry, and J. M. Launay. 1993. A new point mutation in the prion protein gene at codon 210 in Creutzfeldt-Jakob disease. *Neurology* 43:1934-1938.

Robakis, N. K., P. R. Sawh, G. C. Wolfe, R. Rubenstein, R. I. Carp, M. A. Innis. 1986. Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues. *Proceedings of the National Academy of Sciences of the United States of America* 83:6377-6381.

Roemer, K., P. A. Johnson, and T. Friedmann. 1995. Transduction of foreign regulatory sequences by a replication- defective herpes simplex virus type 1: the rat neuron-specific enolase promoter. *Virus Research* 35:81-89.

Rogers, M., A. Tarabolous, M. Scott, D. Groth, and S. B. Prusiner. 1990. Intracellular accumulation of the cellular prion protein after mutagenesis of its Asn-linked glycosylation sites. *Glycobiology* 1:101-109.

Rogers, M., D. Serban, T. Gyuris, M. Scott, T. Torchia, and S. B. Prusiner. 1991. Epitope mapping of the syrian hamster prion protein utilizing chimeric and mutant genes in a vaccinia virus expression system. *Journal of Immunology* 147:3568-3574.

Rogers, M., F. Yehiely, M. Scott, and S. B. Prusiner. 1993. Conversion of truncated and elongated prion proteins into the scrapie isoform in cultured cells. *Proceedings of the National Academy of Sciences of the United States of America* 90:3182-3186.

Rohwer, R. G. 1984. Scrapie infectious agent is virus-like in size and susceptibility to inactivation. *Nature* 308:658-662.

Rubenstein, R., R. I. Carp, and S. M. Callahan. 1984. In vitro replication of scrapie agent in a neuronal model: infection of PC12 cells. *Journal of General Virology* 65:2191-2198.

Rubenstein, R., H. Deng, C. L. Scalici, and M. C. Papini. 1991. Alterations in neurotransmitter-related enzyme activity in scrapie- infected PC12 cells. *Journal of General Virology* 72:1279-1285.

Rubenstein, R., H. Deng, R. E. Race, W. Ju, C. L. Scalici, M. C. Papini, R. J. Kascsak, and R. I. Carp. 1992. Demonstration of scrapie strain diversity in infected PC12 cells. *Journal of General Virology* 73:3027-3031.

Rubenstein, R., H. Deng, R. Race, W. Ju, C. Scalici, M. Papini, A. Rubenstein, R. Kascsak, and R. Carp. 1994. Scrapie strain infection in vitro induces changes in neuronal cells. *Molecular Neurobiology* 8(2-3):129-138.

Sachs, A. B. 1993. Messenger RNA degradation in eukaryotes. *Cell* 74:413-421.

Safar, J., P. P. Roller, D. C. Gajdusek, and J. Gibbs Cj. 1993. Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. *Journal of Biological Chemistry* 268:20276-20284.

Sakimura, K., E. Kushiya, Y. Takahashi, and Y. Suzuki. 1987. The structure and expression of neuron-specific enolase gene. *Gene* 60:103-113.

Sakimura, K., E. Kushiya, A. Ogura, Y. Kudo, T. Katagiri, and Y. Takahashi. 1995. Upstream and intron regulatory regions for expression of the rat neuron-specific enolase gene. *Molecular Brain Research* 28:19-28.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74:5463-5467.

Schmechel, D. E., M. W. Brightman, and P. J. Marangos. 1980. Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. *Brain Research* 190:195-214.

Scott, J. R. 1993. Scrapie pathogenesis. *British Medical Bulletin* 49:778-791.

Scott, M., D. Foster, C. Mirenda, D. Serban, F. Coufal, M. Walchli, M. Torchia, D. Groth, G. Carlson, S. J. DeArmond, D. Westaway, and S. B. Prusiner. 1989. Transgenic mice expressing hamster prion protein produce species- specific scrapie infectivity and amyloid plaques. *Cell* 59:847-857.

Scott, M., D. Groth, D. Foster, M. Torchia, S. L. Yang, S. J. DeArmond, and S. B. Prusiner. 1993. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. *Cell* 73:979-988.

Scott, M. R., R. Kohler, D. Foster, and S. B. Prusiner. 1992. Chimeric prion protein expression in cultured cells and transgenic mice. *Protein Science* 1:986-997.

Scott, M. R. D., D. A. Butler, D. E. Bredesen, M. Walchli, K. K. Hsiao, and S. B. Prusiner. 1988. Prion protein gene expression in cultured cells. *Protein Engineering* 2:69-76.

Seed, B. and J. Y. Sheen. 1988. A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene* 67:271-277.

Sigurdsson, B. 1954. Rida, a chronic encephalitis of sheep: with general remarks on infections which develop slowly and some of their special characteristics. *Brain Veterinary Journal* 110:341-354.

Sklaviadis, T., A. Akowitz, E. E. Manuelidis, and L. Manuelidis. 1990. Nuclease treatment results in high specific purification of Creutzfeldt-Jakob disease infectivity with a density characteristic of nucleic acid-protein complexes. *Archives of Virology* 112:215-228.

Smith, C. and J. Collinge. 1995. Molecular pathology of prion diseases. *Essays in Biochemistry* 29:157-174.

Somerville, R. A. and L. A. Ritchie. 1990. Differential glycosylation of the protein (PrP) forming scrapie- associated fibrils. *Journal of General Virology* 71:833-839.

Sonenberg, N. 1994. mRNA translation: infuence of 5' and 3' untranslated regions. *Current Opinion in Genetics and Development* 4:310-315.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 98:503-505.

Sparkes, R. S., M. Simon, V. H. Cohn, R. E. Fournier, J. Lem, C. Heinzmann, C. Blatt, M. Lucero, T. Mohandas, and et al. 1986. Assignment of the human and mouse prion protein genes to homologous chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* 83:7358-7362.

Stahl, N., D. R. Borchelt, K. K. Hsiao, and S. B. Prusiner. 1987. Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* 51:229-240.

Stahl, N., M. A. Baldwin, A. L. Burlingame, and S. B. Prusiner. 1990a. Identification of glycoinositol phospholipid linked and truncated forms of the scrapie prion protein. *Biochemistry (USA)* 29:8879-8884.

Stahl, N., D. R. Borchelt, and S. B. Prusiner. 1990b. Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. *Biochemistry (USA)* 29:5405-5412.

Stahl, N., D. R. Borchelt, and S. B. Prusiner. 1990c. Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase. *Biochemistry* 29:5405-5412.

Stahl, N., M. A. Baldwin, R. Hecker, K. M. Pan, A. L. Burlingame, and S. B. Prusiner. 1992. Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry (USA)* 31:5043-5053.

Stahl, N., M. A. Baldwin, D. B. Teplow, L. Hood, B. W. Gibson, A. L. Burlingame, and S. B. Prusiner. 1993. Structural studies of the scrapie prion protein using mass spectrometry and amino acid sequencing. *Biochemistry (USA)* 32:1991-2002.

Stamp, J. T., J. G. Brotherston, I. Zlotnik, J. M. K. Mackay, and W. Smith. 1959. Further studies on scrapie. *Journal of Comparative Pathology* 69:268-279.

Stripecke, R., C. C. Oliveira, J. E. G. McCarthy, and M. W. Hentze. 1994. Proteins binding to 5' untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. *Molecular and Cellular Biology* 5898-5909.

Sulkowski, E. 1992. Aromatic palindrome motif in prion proteins. *Faseb Journal* 6:2363.

Sung Ok, Y. and D. M. Chikaraishi. 1992. Tissue-specific transcription of the rat tyrosine hydroxylase gene requires synergy between AP-1 motif and an overlapping e box- containing dyad. *Neuron* 9:55-67.

Tagliavini, F., F. Prelli, J. Ghiso, O. Bugiani, D. Serban, S. B. Prusiner, M. R. Farlow, B. Ghetti, and B. Frangione. 1991. Amyloid protein of Gerstmann-Sträussler-Scheinker disease (Indiana kindred) is an 11 kd fragment of prion protein with an N-terminal glycine at codon 58. *EMBO Journal* 10:513-519.

Tagliavini, F., F. Prelli, M. Porro, M. Salmona, O. Bugiani, and B. Frangione. 1992. A soluble form of prion protein in human cerebrospinal fluid: implications for prion-related encephalopathies. *Biochemical and Biophysical Research Communications* 184:1398-1404.

Tagliavini, F., F. Prelli, M. Porro, G. Rossi, G. Giaccone, M. R. Farlow, S. R. Dlouhy, B. Ghetti, O. Bugiani, and B. Frangione. 1994. Amyloid fibrils in Gerstmann-Sträussler-Scheinker disease (Indiana and Swedish kindreds) express only PrP peptides encoded by the mutant allele. *Cell* 79:695-703.

Taraboulos, A., M. Rogers, D. R. Borchelt, M. P. McKinley, M. Scott, D. Serban, and S. B. Prusiner. 1990a. Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proceedings of the National Academy of Sciences of the United States of America* 87:8262-8266.

Taraboulos, A., D. Serban, and S. B. Prusiner. 1990b. Scrapie prion proteins accumulate in the cytoplasm of persistently infected cultured cells. *J. Cell. Biol.* 110:2117-2132.

Taraboulos, A., K. Jendroska, D. Serban, S. L. Yang, S. J. DeArmond, and S. B. Prusiner. 1992a. Regional mapping of prion proteins in brain. *Proceedings of the National Academy of Sciences of the United States of America* 89:7620-7624.

Taraboulos, A., A. J. Raeber, D. R. Borchelt, D. Serban, and S. B. Prusiner. 1992b. Synthesis and trafficking of prion proteins in cultured cells. *Molecular Biology of the Cell* 3:851-863.

Tateishi, J. and T. Kitamoto. 1993. Developments in diagnosis for prion diseases. *British Medical Bulletin* 49:971-979.

Tateishi, J. and T. Kitamoto. 1995. Inherited prion diseases and transmission to rodents. *Brain Pathology* 5:53-59.

Tateishi,J., P. Brown, T. Kitamoto, Z. M. Hoque, R. Ross, R. Woolman and L. Cervenakova. 1995. First experimental transmission of fatal familial insomnia. *Nature* 376:434-435.

Telling, G. C., M. Scott, K. K. Hsiao, D. Foster, S. L. Yang, M. Torchia, K. C. L. Sidle, J. Collinge, S. J. DeArmond, and S. B. Prusiner. 1994. Transmission of Creutzfeldt-Jakob disease from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proceedings of the National Academy of Sciences of the United States of America* 91:9936-9940.

Telling, G. C., M. Scott, J. Mastrianni, R. Gabizon, M. Torchia, F. E. Cohen, S. J. DeArmond, and S. B. Prusiner. 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell* 83:79-90.

Turk, E., D. B. Teplow, L. E. Hood, and S. B. Prusiner. 1988. Purification and properties of the cellular and scrapie hamster prion proteins. *European Journal of Biochemistry* 176:21-30.

Ulman, A., F. Jacob, and J. Monod. 1967. Characterisation by in vitro complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of echerichia coli . *Journal of Molecular Biology* 24:339-343.

Weissmann, C. 1991. A 'unified theory' of prion propagation. *Nature* 352:679-683.

Weissmann, C., H. Bueler, A. Sailer, M. Fisher, M. Aguet, and A. Aguzzi. 1993. Role of PrP in prion diseases. *British Medical Bulletin* 49:995-1011.

Wells, G. A. H., A. C. Scott, C. T. Johnson, R. F. Gunning, R. D. Hancock, M. Jeffrey, and R. Bradley. 1987. A progressive spongiform encephalopathy in cattle. *Veterinary record* 121:419-420.

Westaway, D. and S. B. Prusiner. 1986. Conservation of the cellular gene encoding the scrapie prion protein. *Nucleic Acids Research* 14:2035-2044.

Westaway, D., P. A. Goodman, C. A. Mirenda, M. P. McKinley, G. A. Carlson, and S. B. Prusiner. 1987. Distinct prion proteins in short and long scrapie incubation period mice. *Cell* 51:651-662.

Westaway, D., C. A. Mirenda, D. Foster, Y. Zebarjadian, M. Scott, M. Torchia, S. L. Yang, H. Serban, S. J. DeArmond, C. Ebeling, S. B. Prusiner, and G. A. Carlson. 1991. Paradoxical shortening of scrapie incubation times by expression of prion protein transgenes derived from long incubation period mice. *Neuron* 7:59-68.

Westaway, D., S. J. DeArmond, J. CayetanoCanlas, D. Groth, D. Foster, L. Yang S, M. Torchia, G. A. Carlson, and S. B. Prusiner. 1994a. Degeneration of skeletal muscle, peripheral nerves, and the central

nervous system in transgenic mice overexpressing wild-type prion proteins. *Cell* 76:117-129.

Westaway, D., V. Zuliani, C. M. Cooper, M. Da Costa, S. Neuman, A. L. Jenny, L. Detwiler, and S. B. Prusiner. 1994b. Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. *Genes and development* 8:959-969.

Whittington, M. A., K. C. L. Sidle, I. Gowland, J. Meads, A. F. Hill, M. S. Palmer, J. G. R. Jefferys, and J. Collinge. 1995. Rescue of neurophysiological phenotype seen in PrP null mice by transgene encoding human prion protein. *Nature genetics* 9:197-201.

Wilesmith, J. W., G. A. H. Wells, M. P. Cranwell, and J. B. M. Ryan. 1988. Bovine spongiform encephalopathy: epidimiological studies. *Veterinary record* 123:638-644.

Wilesmith, J. W., J. B. M. Ryan, and M. J. Atkinson. 1991. Bovine spongiform encephalopatrhy: epidimiological studies on the origin. *Veterinary record* 128:199-203.

Wilesmith, J. W., J. B. M. Ryan, W. D. Hueston, and L. J. Hoinville. 1992. Bovine spongiform encephalopathy: epidimiological features 1985 to 1990. *Veterinary record* 130:90-94.

Will, R. G. and W. B. Matthews. 1984. A retrospective study of Creutzfeldt-Jacob disease in England and Wales 1970-79. I: Clinical features. *Journal of Neurology Neurosurgery and Psychiatry* 47:134-140.

Will, R. G. 1993. Epidimiology of Creutzfeldt-Jakob disease. *British Medical Bulletin* 49:960-970.

Williams, E. S. and S. Young. 1980. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *Journal of Wildlife Diseases* 16:89-98.

Williams, E. S. and S. Young. 1982. Spongiform encephalopathy of Rocky Mountain elk. *Journal of Wildlife Diseases* 18:465-471.

Willoughby, K., D. F. Kelly, D. G. Lyon, and G. A. Wells. 1992. Spongiform encephalopathy in captive puma (Felis concolor). *Veterinary record* 131:431-434.

Windl, O., M. Dempster, P. Estibeiro, and R. Lathe. 1995. A candidate marsupial PrP gene reveals two domains conserved in mammalian PrP proteins. *Gene* 159:181-186.

Windl, O., M. Dempster, J. P. Estibeiro, R. Lathe, R. de Silva, *et al.* 1996. Genetic basis of Creutzfeldt-Jakob disease in the United Kingdom: a systematic analysis of predisposing mutations and allelic variation in the PRNP gene. Human Genetics (submitted).

Wion, D., M. Le Bert, and P. Brachet. 1988. Messsenger RNAs of β-amyloid precursor protein and prion protein are regulated by nerve growth factor in PC12 cells. *International Journal of Developments in Neuroscience* 6:387-393.

Wood, J. L. N. and S. H. Done. 1992. Natural scrapie in goats: neuropathology. *Veterinary record* 131:93-96.

Wyatt, J. M., G. R. Pearson, T. N. Smerdon, T. J. Gruffydd, G. A. Wells, and J. W. Wilesmith. 1991. Naturally occuring scrapie-like spongiform encephalopathy in five domestic cats. *Veterinary record* 129:233-236.

Yoshikawa, K., T. Aizawa, and Y. Hayashi. 1992. Degeneration in vitro of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor . *Nature* 359:64-67.

Yost, C. S., C. D. Lopez, S. B. Prusiner, R. M. Myers, and V. R. Lingappa. 1990. Non-hydrophobic extracytoplasmic determinant of stop transfer in the prion protein. *Nature* 343:669-672.

Zlotnik, I. 1958. The histopathology of the brain stem of sheep affected with natural scrapie. *Journal of Comparative Pathology* 68:148-165.

Zlotnik, I. and J. C. Rennie. 1962. The pathology of the brain of mice inoculated with tissues from scrapie sheep. *Journal of Comparative Pathology* 72:360-365.